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(54) Title: ELECTRONIC METHODS FOR THE DETECTION OF ANALYTES UTILIZING MONOLAYERS

(57) Abstract

The present invention relates to the use of self–assembled monolayers with mixtures of conductive oligomers and insulators to detect target analytes.

*(Referred to in PCT Gazette No. 7/2000, Section II)

ELECTRONIC METHODS FOR THE DETECTION OF ANALYTES UTILIZING MONOLAYERS

This application is a continuing application of U.S.S.N.s 60/084,652, filed May 6, 1998; 60/084,509, filed May 6, 1998, and 09/135,183, filed August 17, 1998.

FIELD OF THE INVENTION

The present invention relates to the use of self-assembled monolayers with mixtures of conductive oligomers and insulators to detect target analytes.

BACKGROUND OF THE INVENTION

There are a number of assays and sensors for the detection of the presence and/or concentration of specific substances in fluids and gases. Many of these rely on specific ligand/antiligand reactions as the mechanism of detection. That is, pairs of substances (i.e. the binding pairs or ligand/antiligands) are known to bind to each other, while binding little or not at all to other substances. This has been the focus of a number of techniques that utilize these binding pairs for the detection of the complexes. These generally are done by labeling one component of the complex in some way, so as to make the entire complex detectable, using, for example, radioisotopes, fluorescent and other optically active molecules, enzymes, etc.

Other assays rely on electronic signals for detection. Of particular interest are biosensors. At least two types of biosensors are known; enzyme-based or metabolic biosensors and binding or bioaffinity sensors. See for example U.S. Patent No. 4,713,347; 5,192,507; 4,920,047; 3,873,267; and references disclosed therein. While some of these known sensors use alternating current (AC)

120 and a second portion that will hybridize to the capture probe 100. Figure 1C depicts the use of two capture extender probes 110 and 130. The first capture extender probe 110 has a first portion 111 that will hybridize to a first portion of the target sequence 120 and a second portion 112 that will hybridize to a first portion 102 of the capture probe 100. The second capture extender probe 130 has a first portion 132 that will hybridize to a second portion of the target sequence 120 and a second portion 131 that will hybridize to a second portion 101 of the capture probe 100. As will be appreciated by those in the art, while these systems depict nucleic acid targets, these attachment configurations may be used with non-nucleic acid capture binding ligands; see for example Figure 2C.

Figure 2A, 2B, 2C and 2D depict several embodiments of the invention. Figure 2A is directed to the use of a capture binding ligand 200 attached via an attachment linker 106 to the electrode 105. Target analyte 210 binds to the capture binding ligand 200, and a solution binding ligand 22 with a directly attached recruitment linker 230 with ETMs 135. Figure 2B depicts a similar embodiment using an indirectly attached recruitment linker 145 that binds to a second portion 240 of the solution binding ligand 220. Figure 2C depicts the use of an anchor ligand 100 (referred to herein as an anchor probe when the ligand comprises nucleic acid) to bind the capture binding ligand 200 comprising a portion 120 that will bind to the anchor probe 100. As will be appreciated by those in the art, any of the Figure 1 embodiments may be used here as well. Figure 2D depicts the use of an amplifier probe 145. As will be appreciated by those in the art, any of the Figure 3 amplifier probe configurations may be used here as well.

Figures 3A, 3B, 3C, 3D, 3E, 3F, 3G and 3H depict some of the embodiments of the invention. While depicted for nucleic acids, they can be used in non-nucleic acid embodiments as well. All of the monolayers depicted herein show the presence of both conductive oligomers 108 and insulators 107 in roughly a 1:1 ratio, although as discussed herein, a variety of different ratios may be used, or the insulator may be completely absent. In addition, as will be appreciated by those in the art, any one of these structures may be repeated for a particular target sequence; that is, for long target sequences, there may be multiple assay complexes formed. Additionally, any of the electrode-attachment embodiments of Figure 3 may be used in any of these systems.

Figures 3A, 3B and 3D have the target sequence 120 containing the ETMs 135; as discussed herein, these may be added enzymatically, for example during a PCR reaction using nucleotides modified with ETMs, resulting in essentially random incorporation throughout the target sequence, or added to the terminus of the target sequence. Figure 3C depicts the use of two different capture probes 100 and 100', that hybridize to different portions of the target sequence 120. As will be appreciated by those in the art, the 5'-3' orientation of the two capture probes in this embodiment is different.

binding ligands **200**; these may also be attached to the electrode using capture extender ligands. Figure 5E adds an additional "sandwich component" in the form of an additional solution binding ligand **250**.

Figures 6A-6R depict nucleic acid detection systems. Figures 6A and 6B have the target sequence **5** containing the ETMs **6**; as discussed herein, these may be added enzymatically, for example during a PCR reaction using nucleotides modified with ETMs, resulting in essentially random incorporation throughout the target sequence, or added to the terminus of the target sequence. Figure 6A shows attachment of a capture probe **10** to the electrode **20** via a linker **15**, which as discussed herein can be either a conductive oligomer **25** or an insulator **30**. The target sequence **5** contains ETMs **6**. Figure 6B depicts the use of a capture extender probe **11**, comprising a first portion **12** that hybridizes to a portion of the target sequence and a second portion **13** that hybridizes to the capture probe **10**.

Figure 6C depicts the use of two different capture probes **10** and **10'**, that hybridize to different portions of the target sequence **5**. As will be appreciated by those in the art, the 5'-3' orientation of the two capture probes in this embodiment is different.

Figures 6D to 6H depict the use of label probes **40** that hybridize directly to the target sequence **5**. Figure 6D shows the use of a label probe **40**, comprising a first portion **41** that hybridizes to a portion of the target sequence **5**, a second portion **42** that hybridizes to the capture probe **10** and a recruitment linker **50** comprising ETMs **6**. A similar embodiment is shown in Figure 6E, where the label probe **40** has an additional recruitment linker **50**. Figure 6F depicts a label probe **40** comprising a first portion **41** that hybridizes to a portion of the target sequence **5** and a recruitment linker **50** with attached ETMs **6**. The parentheses highlight that for any particular target sequence **5** more than one label probe **40** may be used, with n being an integer of at least 1. Figure 6G depicts the use of the Figure 6E label probe structures but includes the use of a single capture extender probe **11**, with a first portion **12** that hybridizes to a portion of the target sequence and a second portion **13** that hybridizes to the capture probe **10**. Figure 6H depicts the use of the Figure 6F label probe structures but utilizes two capture extender probes **11** and **16**. The first capture extender probe **11** has a first portion **12** that hybridizes to a portion of the target sequence **5** and a second portion **13** that hybridizes to a first portion **14** of the capture probe **10**. The second capture extender probe **16** has a first portion **18** that hybridizes to a second portion of the target sequence **5** and a second portion **17** that hybridizes to a second portion **19** of the capture probe **10**.

Figures 6I, 6J and 6K depict systems utilizing label probes **40** that do not hybridize directly to the target, but rather to amplifier probes. Thus the amplifier probe **60** has a first portion **65** that hybridizes

Figure 13 depicts the synthesis of an insulator to the ribose of a nucleoside for attachment to an electrode.

Figures 14A, 14B, 14C, 14D, 14E, 14F, 14G, 14H, 14I, 14J and 14K depict a number of different embodiments of the invention; the results are shown in Example 7.

Figures 15A-15O depict depict a number of different compositions of the invention; the results are shown in Example 7 and 8. Figure 15A depicts **I**, also referred to as **P290**. Figure 15B depicts **II**, also referred to as **P291**. Figure 15C depicts **III**, also referred to as **W31**. Figure 15D depicts **IV**, also referred to as **N6**. Figure 15E depicts **V**, also referred to as **P292**. Figure 51F depicts **II**, also referred to as **C23**. Figure 15G depicts **VII**, also referred to as **C15**. Figure 15H depicts **VIII**, also referred to as **C95**. Figure 15I depicts **Y63**. Figure 1J depicts another compound of the invention. Figure 15K depicts **N11**. Figure 15L depicts **C131**, with a phosphoramidite group and a DMT protecting group. Figure 15M depicts **W38**, also with a phosphoramidite group and a DMT protecting group. Figure 15N depicts the commercially available moiety that enables "branching" to occur, as its incorporation into a growing oligonucleotide chain results in addition at both the DMT protected oxygens. Figure 15O depicts **glen**, also with a phosphoramidite group and a DMT protecting group, that serves as a non-nucleic acid linker. Figures 15A to 15G and 15J are shown without the phosphoramidite and protecting groups (i.e. DMT) that are readily added.

Figures 16A - 16B depict representative scans from the experiments outlined in Example 7. Unless otherwise noted, all scans were run at initial voltage -0.11 V, final voltage 0.5 V, with points taken every 10 mV, amplitude of 0.025, frequency of 10 Hz, a sample period of 1 sec, a quiet time of 2 sec. Figure 16A has a peak potential of 0.160 V, a peak current of 1.092×10^{-8} A, and a peak A of 7.563×10^{-10} VA.

Figure 17 depicts the synthetic scheme for a ribose linked ETM, W38.

Figures 18A and 18B depicts two phosphate attachments of conductive oligomers that can be used to add the conductive oligomers at the 5' position, or any position.

Figure 19 depicts a schematic of the synthesis of simultaneous incorporation of multiple ETMs into a nucleic acid, using a "branch" point nucleoside.

recruitment of ETMs to the surface, where they can be detected using the electrode. Without being bound by theory, one possible mechanism is that the role of the SAM comprising the conductive oligomers is to "raise" the electronic surface of the electrode, while still providing the benefits of shielding the electrode from solution components and reducing the amount of non-specific binding to the electrodes.

The invention can be generally described as follows, with a number of possible embodiments depicted in the Figures. In a preferred embodiment, as depicted in Figure 2, an electrode comprising a self-assembled monolayer (SAM) of conductive oligomers, and preferably a mixture of conductive oligomers and insulators, and a covalently attached target analyte binding ligand (frequently referred to herein as a "capture binding ligand") is made. The target analyte is added, which binds to the support-bound binding ligand. A solution binding ligand is added, which may be the same or different from the first binding ligand, which can also bind to the target analyte, forming a "sandwich" of sorts. The solution binding ligand either comprises a recruitment linker containing ETMs, or comprises a portion that will either directly or indirectly bind a recruitment linker containing the ETMs. This "recruitment" of ETMs to the surface of the monolayer allows electronic detection via electron transfer between the ETM and the electrode. In the absence of the target analyte, the recruitment linker is either washed away or not in sufficient proximity to the surface to allow detection.

In an alternate preferred embodiment, as depicted in Figure 4, a competitive binding type assay is run. In this embodiment, the target analyte in the sample is replaced by a target analyte analog as is described below and generally known in the art. The analog comprises a directly or indirectly attached recruitment linker comprising at least one ETM. The binding of the analog to the capture binding ligand recruits the ETM to the surface and allows detection based on electron transfer between the ETM and the electrode.

In an additional preferred embodiment, as depicted in Figure 4B, a competitive assay wherein the target analyte and a target analyte analog attached to the surface compete for binding of a solution binding ligand with a directly or indirectly attached recruitment linker. In this case, a loss of signal may be seen.

Accordingly, the present invention provides methods and compositions useful in the detection of target analytes. By "target analyte" or "analyte" or grammatical equivalents herein is meant any molecule or compound to be detected and that can bind to a binding species, defined below. Suitable analytes include, but not limited to, small chemical molecules such as environmental or clinical chemical or pollutant or biomolecule, including, but not limited to, pesticides, insecticides, toxins, therapeutic and abused drugs, hormones, antibiotics, antibodies, organic materials, etc. Suitable biomolecules

Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of ETMs, or to increase the stability and half-life of such molecules in physiological environments.

As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; for example, at the site of conductive oligomer or ETM attachment, an analog structure may be used. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occuring nucleic acids and analogs may be made.

Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (Tm) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4°C drop in Tm for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9°C. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration. This is particularly advantageous in the systems of the present invention, as a reduced salt hybridization solution has a lower Faradaic current than a physiological salt solution (in the range of 150 mM).

The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. A preferred embodiment utilizes isocytosine and isoguanine in nucleic acids designed to be complementary to other probes, rather than target sequences, as this reduces non-specific hybridization, as is generally described in U.S. Patent No. 5,681,702. As used herein, the term "nucleoside" includes nucleotides as well as nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occuring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

maintenance of the target analyte away from the electrode surface. In addition, a monolayer serves to keep charge carriers away from the surface of the electrode. Thus, this layer helps to prevent electrical contact between the electrodes and the ETMs, or between the electrode and charged species within the solvent. Such contact can result in a direct "short circuit" or an indirect short circuit via charged species which may be present in the sample. Accordingly, the monolayer is preferably tightly packed in a uniform layer on the electrode surface, such that a minimum of "holes" exist. The monolayer thus serves as a physical barrier to block solvent accesibility to the electrode.

In a preferred embodiment, the monolayer comprises conductive oligomers. By "conductive oligomer" herein is meant a substantially conducting oligomer, preferably linear, some embodiments of which are referred to in the literature as "molecular wires". By "substantially conducting" herein is meant that the oligomer is capable of transfering electrons at 100 Hz. Generally, the conductive oligomer has substantially overlapping π-orbitals, i.e. conjugated π-orbitals, as between the monomeric units of the conductive oligomer, although the conductive oligomer may also contain one or more sigma (σ) bonds. Additionally, a conductive oligomer may be defined functionally by its ability to inject or receive electrons into or from an associated ETM. Furthermore, the conductive oligomer is more conductive than the insulators as defined herein. Additionally, the conductive oligomers of the invention are to be distinguished from electroactive polymers, that themselves may donate or accept electrons.

In a preferred embodiment, the conductive oligomers have a conductivity, S, of from between about 10^{-6} to about 10^{4} $\Omega^{-1}cm^{-1}$, with from about 10^{-5} to about 10^{3} $\Omega^{-1}cm^{-1}$ being preferred, with these S values being calculated for molecules ranging from about 20Å to about 200Å. As described below, insulators have a conductivity S of about 10^{-7} $\Omega^{-1}cm^{-1}$ or lower, with less than about 10^{-8} $\Omega^{-1}cm^{-1}$ being preferred. See generally Gardner et al., Sensors and Actuators A 51 (1995) 57-66, incorporated herein by reference.

Desired characteristics of a conductive oligomer include high conductivity, sufficient solubility in organic solvents and/or water for synthesis and use of the compositions of the invention, and preferably chemical resistance to reactions that occur i) during nucleic acid synthesis (such that nucleosides containing the conductive oligomers may be added to a nucleic acid synthesizer during the synthesis of the compositions of the invention), ii) during the attachment of the conductive oligomer to an electrode, or iii) during hybridization assays. In addition, conductive oligomers that will promote the formation of self-assembled monolayers are preferred.

The oligomers of the invention comprise at least two monomeric subunits, as described herein. As is described more fully below, oligomers include homo- and hetero-oligomers, and include polymers.

Importantly, the Y aromatic groups of the conductive oligomer may be different, i.e. the conductive oligomer may be a heterooligomer. That is, a conductive oligomer may comprise a oligomer of a single type of Y groups, or of multiple types of Y groups.

The aromatic group may be substituted with a substitution group, generally depicted herein as R. R groups may be added as necessary to affect the packing of the conductive oligomers, i.e. R groups may be used to alter the association of the oligomers in the monolayer. R groups may also be added to 1) alter the solubility of the oligomer or of compositions containing the oligomers; 2) alter the conjugation or electrochemical potential of the system; and 3) alter the charge or characteristics at the surface of the monolayer.

In a preferred embodiment, when the conductive oligomer is greater than three subunits, R groups are preferred to increase solubility when solution synthesis is done. However, the R groups, and their positions, are chosen to minimally effect the packing of the conductive oligomers on a surface, particularly within a monolayer, as described below. In general, only small R groups are used within the monolayer, with larger R groups generally above the surface of the monolayer. Thus for example the attachment of methyl groups to the portion of the conductive oligomer within the monolayer to increase solubility is preferred, with attachment of longer alkoxy groups, for example, C3 to C10, is preferably done above the monolayer surface. In general, for the systems described herein, this generally means that attachment of sterically significant R groups is not done on any of the first two or three oligomer subunits, depending on the average length of the molecules making up the monolayer.

Suitable R groups include, but are not limited to, hydrogen, alkyl, alcohol, aromatic, amino, amido, nitro, ethers, esters, aldehydes, sulfonyl, silicon moieties, halogens, sulfur containing moieties, phosphorus containing moieties, and ethylene glycols. In the structures depicted herein, R is hydrogen when the position is unsubstituted. It should be noted that some positions may allow two substitution groups, R and R', in which case the R and R' groups may be either the same or different.

By "alkyl group" or grammatical equivalents herein is meant a straight or branched chain alkyl group, with straight chain alkyl groups being preferred. If branched, it may be branched at one or more positions, and unless specified, at any position. The alkyl group may range from about 1 to about 30 carbon atoms (C1 -C30), with a preferred embodiment utilizing from about 1 to about 20 carbon atoms (C1 -C20), with about C1 through about C12 to about C15 being preferred, and C1 to C5 being particularly preferred, although in some embodiments the alkyl group may be much larger. Also included within the definition of an alkyl group are cycloalkyl groups such as C5 and C6 rings, and heterocyclic rings with nitrogen, oxygen, sulfur or phosphorus. Alkyl also includes heteroalkyl, with heteroatoms of sulfur, oxygen, nitrogen, and silicone being preferred. Alkyl includes substituted alkyl

Preferred aromatic groups include, but are not limited to, phenyl, naphthyl, naphthalene, anthracene, phenanthroline, pyrole, pyridine, thiophene, porphyrins, and substituted derivatives of each of these, included fused ring derivatives.

In the conductive oligomers depicted herein, when g is 1, B-D is a bond linking two atoms or chemical moieties. In a preferred embodiment, B-D is a conjugated bond, containing overlapping or conjugated π-orbitals.

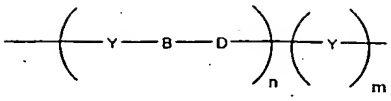
Preferred B-D bonds are selected from acetylene (-C≡C-, also called alkyne or ethyne), alkene (-CH=CH-, also called ethylene), substituted alkene (-CR=CR-, -CH=CR- and -CR=CH-), amide (-NH-CO- and -NR-CO- or -CO-NH- and -CO-NR-), azo (-N=N-), esters and thioesters (-CO-O-, -O-CO-, -CS-O- and -O-CS-) and other conjugated bonds such as (-CH=N-, -CR=N-, -N=CH- and -N=CR-), (-SiH=SiH-, -SiR=SiH-, -SiR=SiH-, and -SiR=SiR-), (-SiH=CH-, -SiR=CH-, -SiH=CR-, -SiR=CR-, -CH=SiH-, -CR=SiH-, -CH=SiR-, and -CR=SiR-). Particularly preferred B-D bonds are acetylene, alkene, amide, and substituted derivatives of these three, and azo. Especially preferred B-D bonds are acetylene, alkene and amide. The oligomer components attached to double bonds may be in the trans or cis conformation, or mixtures. Thus, either B or D may include carbon, nitrogen or silicon. The substitution groups are as defined as above for R.

When g=0 in the Structure 1 conductive oligomer, e is preferably 1 and the D moiety may be carbonyl or a heteroatom moiety as defined above.

As above for the Y rings, within any single conductive oligomer, the B-D bonds (or D moieties, when g=0) may be all the same, or at least one may be different. For example, when m is zero, the terminal B-D bond may be an amide bond, and the rest of the B-D bonds may be acetylene bonds. Generally, when amide bonds are present, as few amide bonds as possible are preferable, but in some embodiments all the B-D bonds are amide bonds. Thus, as outlined above for the Y rings, one type of B-D bond may be present in the conductive oligomer within a monolayer as described below, and another type above the monolayer level, for example to give greater flexibility for analyte - binding ligand binding, when the capture binding ligand is attached via a conductive oligomer.

In the structures depicted herein, n is an integer from 1 to 50, although longer oligomers may also be used (see for example Schumm et al., Angew. Chem. Int. Ed. Engl. 1994 33(13):1360). Without being bound by theory, it appears that for efficient association of binding ligands and targets, the reaction should occur at a distance from the surface. Thus, for example, for nucleic acid hybridization of target nucleic acids to capture probes on a surface, the hybridization should occur at a distance from the surface, i.e. the kinetics of hybridization increase as a function of the distance from the

Structure 3

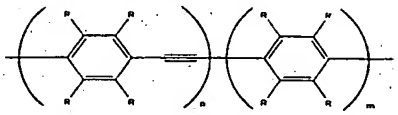


Preferred embodiments of Structure 3 are: Y is phenyl or substituted phenyl and B-D is azo; Y is phenyl or substituted phenyl and B-D is acetylene; Y is phenyl or substituted phenyl and B-D is alkene; Y is pyridine or substituted pyridine and B-D is acetylene; Y is thiophene or substituted thiophene and B-D is acetylene; Y is furan or substituted furan and B-D is acetylene; Y is thiophene or furan (or substituted thiophene or furan) and B-D are alternating alkene and acetylene bonds.

Most of the structures depicted herein utilize a Structure 3 conductive oligomer. However, any Structure 3 oligomers may be substituted with any of the other structures depicted herein, i.e. Structure 1 or 8 oligomer, or other conducting oligomer, and the use of such Structure 3 depiction is not meant to limit the scope of the invention.

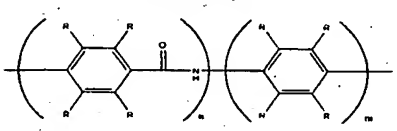
Particularly preferred embodiments of Structure 3 include Structures 4, 5, 6 and 7, depicted below:

Structure 4



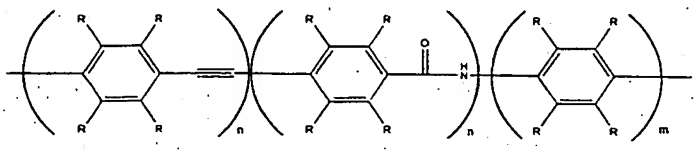
Particularly preferred embodiments of Structure 4 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen; and the use of R groups to increase solubility.

Structure 5



When the B-D bond is an amide bond, as in Structure 5, the conductive oligomers are pseudopeptide oligomers. Although the amide bond in Structure 5 is depicted with the carbonyl to the left, i.e. -CONH-, the reverse may also be used, i.e. -NHCO-. Particularly preferred embodiments of Structure 5 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen (in this embodiment, the terminal nitrogen (the D atom) may be the nitrogen of the amino-modified ribose); and the use of R groups to increase solubility.

Structure 6



The alkene oligomer of structure 9, and others depicted herein, are generally depicted in the preferred trans configuration, although oligomers of cis or mixtures of trans and cis may also be used. As above, R groups may be added to alter the packing of the compositions on an electrode, the hydrophilicity or hydrophobicity of the oligomer, and the flexibility, i.e. the rotational, torsional or longitudinal flexibility of the oligomer. n is as defined above.

In a preferred embodiment, R is hydrogen, although R may be also alkyl groups and polyethylene glycols or derivatives.

In an alternative embodiment, the conductive oligomer may be a mixture of different types of oligomers, for example of structures 1 and 8.

In addition, the terminus of at least some of the conductive oligomers in the monolayer are electronically exposed. By "electronically exposed" herein is meant that upon the placement of an ETM in close proximity to the terminus, and after initiation with the appropriate signal, a signal dependent on the presence of the ETM may be detected. The conductive oligomers may or may not have terminal groups. Thus, in a preferred embodiment, there is no additional terminal group, and the conductive oligomer terminates with one of the groups depicted in Structures 1 to 9; for example, a B-D bond such as an acetylene bond. Alternatively, in a preferred embodiment, a terminal group is added, sometimes depicted herein as "Q". A terminal group may be used for several reasons; for example, to contribute to the electronic availability of the conductive oligomer for detection of ETMs, or to alter the surface of the SAM for other reasons, for example to prevent non-specific binding. For example, there may be negatively charged groups on the terminus to form a negatively charged surface such that when the target analyte is nucleic acid such as DNA or RNA, the nucleic acid is repelled or prevented from lying down on the surface, to facilitate hybridization. Preferred terminal groups include $-NH_2$, -OH, -COOH, and alkyl groups such as $-CH_3$, and (poly)alkyloxides such as (poly)ethylene glycol, with $-OCH_2CH_2OH$, $-(OCH_2CH_2O)_2H$, $-(OCH_2CH_2O)_3H$, and $-(OCH_2CH_2O)_4H$ being preferred.

In one embodiment, it is possible to use mixtures of conductive oligomers with different types of terminal groups. Thus, for example, some of the terminal groups may facilitate detection, and some may prevent non-specific binding.

It will be appreciated that the monolayer may comprise different conductive oligomer species, although preferably the different species are chosen such that a reasonably uniform SAM can be formed. Thus, for example, when capture binding ligands are covalently attached to the electrode using conductive oligomers, it is possible to have one type of conductive oligomer used to attach the capture binding

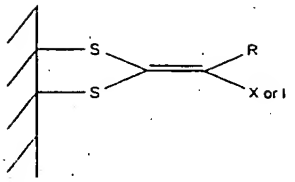
nucleic acids being more accessible to the solvent for hybridization. In some embodiments, the conductive oligomers to which the capture binding ligands are attached may be shorter than the monolayer.

As will be appreciated by those in the art, the actual combinations and ratios of the different species making up the monolayer can vary widely. Generally, three component systems are preferred, with the first species comprising a capture binding ligand containing species (i.e. a capture probe, that can be attached to the electrode via either an insulator or a conductive oligomer, as is more fully described below). The second species are the conductive oligomers, and the third species are insulators. In this embodiment, the first species can comprise from about 90% to about 1%, with from about 20% to about 40% being preferred. When the capture binding ligands are nucleic acids and the target is nucleic acid as well, from about 30% to about 40% is especially preferred for short oligonucleotide targets and from about 10% to about 20% is preferred for longer targets. The second species can comprise from about 1% to about 90%, with from about 20% to about 90% being preferred, and from about 40% to about 60% being especially preferred. The third species can comprise from about 1% to about 90%, with from about 20% to about 40% being preferred, and from about 15% to about 30% being especially preferred. Preferred ratios of first:second:third species are 2:2:1 for short targets, 1:3:1 for longer targets, with total thiol concentration in the 500 μM to 1 mM range, and 833 μM being preferred.

In a preferred embodiment, two component systems are used, comprising the first and second species. In this embodiment, the first species can comprise from about 90% to about 1%, with from about 1% to about 40% being preferred, and from about 10% to about 40% being especially preferred. The second species can comprise from about 1% to about 90%, with from about 10% to about 60% being preferred, and from about 20% to about 40% being especially preferred.

The covalent attachment of the conductive oligomers and insulators may be accomplished in a variety of ways, depending on the electrode and the composition of the insulators and conductive oligomers used. In a preferred embodiment, the attachment linkers with covalently attached capture binding ligands as depicted herein are covalently attached to an electrode. Thus, one end or terminus of the attachment linker is attached to the capture binding ligand, and the other is attached to an electrode. In some embodiments it may be desirable to have the attachment linker attached at a position other than a terminus, or even to have a branched attachment linker that is attached to an electrode at one terminus and to two or more capture binding ligands at other termini, although this is not preferred. Similarly, the attachment linker may be attached at two sites to the electrode, as is generally depicted in Structures 11-13. Generally, some type of linker is used, as depicted below as "A" in Structure 10, where "X" is the conductive oligomer, "I" is an insulator and the hatched surface is the electrode:

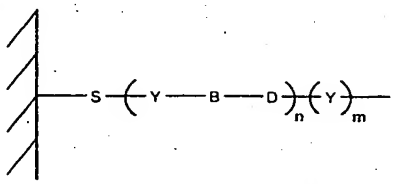
Structure 13



It should also be noted that similar to Structure 13, it may be possible to have a a conductive oligomer terminating in a single carbon atom with three sulfur moities attached to the electrode. Additionally, although not always depicted herein, the conductive oligomers and insulators may also comprise a "Q" terminal group.

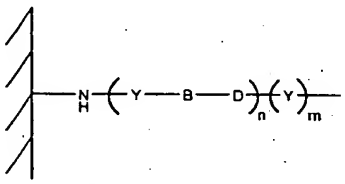
In a preferred embodiment, the electrode is a gold electrode, and attachment is via a sulfur linkage as is well known in the art, i.e. the A moiety is a sulfur atom or moiety. Although the exact characteristics of the gold-sulfur attachment are not known, this linkage is considered covalent for the purposes of this invention. A representative structure is depicted in Structure 14, using the Structure 3 conductive oligomer, although as for all the structures depicted herein, any of the conductive oligomers, or combinations of conductive oligomers, may be used. Similarly, any of the conductive oligomers or insulators may also comprise terminal groups as described herein. Structure 14 depicts the "A" linker as comprising just a sulfur atom, although additional atoms may be present (i.e. linkers from the sulfur to the conductive oligomer or substitution groups).

Structure 14



In a preferred embodiment, the electrode is a carbon electrode, i.e. a glassy carbon electrode, and attachment is via a nitrogen of an amine group. A representative structure is depicted in Structure 15. Again, additional atoms may be present, i.e. Z type linkers and/or terminal groups.

Structure 15



acid; alternatively, the binding ligand may be a nucleic acid-binding protein when the analyte is a single or double-stranded nucleic acid. When the analyte is a protein, the binding ligands include proteins or small molecules. Preferred binding ligand proteins include peptides. For example, when the analyte is an enzyme, suitable binding ligands include substrates and inhibitors. As will be appreciated by those in the art, any two molecules that will associate may be used, either as an analyte or as the binding ligand. Suitable analyte/binding ligand pairs include, but are not limited to, antibodies/antigens, receptors/ligands, proteins/nucleic acid, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids)/lectins, proteins/proteins, proteins/small molecules; and carbohydrates and their binding partners are also suitable analyte-binding ligand pairs. These may be wild-type or derivative sequences. In a preferred embodiment, the binding ligands are portions (particularly the extracellular portions) of cell surface receptors that are known to multimerize, such as the growth hormone receptor, glucose transporters (particularly GLUT 4 receptor), transferrin receptor, epidermal growth factor receptor, low density lipoprotein receptor, high density lipoprotein receptor, epidermal growth factor receptor, leptin receptor, interleukin receptors including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-13, IL-15, and IL-17 receptors, human growth hormone receptor, VEGF receptor, PDGF receptor, EPO receptor, TPO receptor, ciliary neurotrophic factor receptor, prolactin receptor, and T-cell receptors.

The method of attachment of the capture binding ligand to the attachment linker will generally be done as is known in the art, and will depend on the composition of the attachment linker and the capture binding ligand. In general, the capture binding ligands are attached to the attachment linker through the use of functional groups on each that can then be used for attachment. Preferred functional groups for attachment are amino groups, carboxy groups, oxo groups and thiol groups. These functional groups can then be attached, either directly or through the use of a linker, sometimes depicted herein as "Z". Linkers are known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). Preferred Z linkers include, but are not limited to, alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), with short alkyl groups, esters, amide, amine, epoxy groups and ethylene glycol and derivatives being preferred. Z may also be a sulfone group, forming sulfonamide.

In this way, capture binding ligands comprising proteins, lectins, nucleic acids, small organic molecules, carbohydrates, etc. can be added.

In a preferred embodiment, the capture binding ligand is attached directly to the electrode as outlined herein, for example via an attachment linker. Alternatively, the capture binding ligand may utilize a capture extender component, such as depicted in Figure 2C. In this embodiment, the capture binding

In general, the methods, synthetic schemes and compositions useful for the attachment of capture binding ligands, particularly nucleic acids, are outlined in WO98/20162, PCT US98/12430, PCT US98/12082; PCT US99/01705 and PCT US99/01703, all of which are expressly incorporated herein by reference in their entirety.

In a preferred embodiment, the capture binding ligand is covalently attached to the electrode via a conductive oligomer. The covalent attachment of the binding ligand and the conductive oligomer may be accomplished in several ways, as will be appreciated by those in the art.

In a preferred embodiment, the capture binding ligand is a nucleic acid, and the attachment is via attachment to the base of the nucleoside, via attachment to the backbone of the nucleic acid (either the ribose, the phosphate, or to an analogous group of a nucleic acid analog backbone), or via a transition metal ligand, as described below. The techniques outlined below are generally described for naturally occuring nucleic acids, although as will be appreciated by those in the art, similar techniques may be used with nucleic acid analogs.

In a preferred embodiment, the conductive oligomer is attached to the base of a nucleoside of the nucleic acid. This may be done in several ways, depending on the oligomer, as is described below. In one embodiment, the oligomer is attached to a terminal nucleoside, i.e. either the 3' or 5' nucleoside of the nucleic acid. Alternatively, the conductive oligomer is attached to an internal nucleoside.

The point of attachment to the base will vary with the base. Generally, attachment at any position is possible. In some embodiments, for example when the probe containing the ETMs may be used for hybridization, it is preferred to attach at positions not involved in hydrogen bonding to the complementary base. Thus, for example, generally attachment is to the 5 or 6 position of pyrimidines such as uridine, cytosine and thymine. For purines such as adenine and guanine, the linkage is preferably via the 8 position. Attachment to non-standard bases is preferably done at the comparable positions.

In one embodiment, the attachment is direct; that is, there are no intervening atoms between the conductive oligomer and the base. In this embodiment, for example, conductive oligomers with terminal acetylene bonds are attached directly to the base. Structure 18 is an example of this linkage, using a Structure 3 conductive oligomer and uridine as the base, although other bases and conductive oligomers can be used as will be appreciated by those in the art:

In a preferred embodiment, the attachment of the nucleic acid and the conductive oligomer is done via attachment to the backbone of the nucleic acid. This may be done in a number of ways, including attachment to a ribose of the ribose-phosphate backbone, or to the phosphate of the backbone, or other groups of analogous backbones.

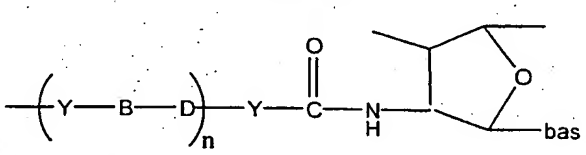
As a preliminary matter, it should be understood that the site of attachment in this embodiment may be to a 3' or 5' terminal nucleotide, or to an internal nucleotide, as is more fully described below.

In a preferred embodiment, the conductive oligomer is attached to the ribose of the ribose-phosphate backbone. This may be done in several ways. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose with amino groups, sulfur groups, silicone groups, phosphorus groups, or oxo groups can be made (Imazawa et al., J. Org. Chem., 44:2039 (1979); Hobbs et al., J. Org. Chem. 42(4):714 (1977); Verheyden et al., J. Orrg. Chem. 36(2):250 (1971); McGee et al., J. Org. Chem. 61:781-785 (1996); Mikhailopulo et al., Liebigs. Ann. Chem. 513-519 (1993); McGee et al., Nucleosides & Nucleotides 14(6):1329 (1995), all of which are incorporated by reference). These modified nucleosides are then used to add the conductive oligomers.

A preferred embodiment utilizes amino-modified nucleosides. These amino-modified riboses can then be used to form either amide or amine linkages to the conductive oligomers. In a preferred embodiment, the amino group is attached directly to the ribose, although as will be appreciated by those in the art, short linkers such as those described herein for "Z" may be present between the amino group and the ribose.

In a preferred embodiment, an amide linkage is used for attachment to the ribose. Preferably, if the conductive oligomer of Structures 1-3 is used, m is zero and thus the conductive oligomer terminates in the amide bond. In this embodiment, the nitrogen of the amino group of the amino-modified ribose is the "D" atom of the conductive oligomer. Thus, a preferred attachment of this embodiment is depicted in Structure 20 (using the Structure 3 conductive oligomer):

Structure 20

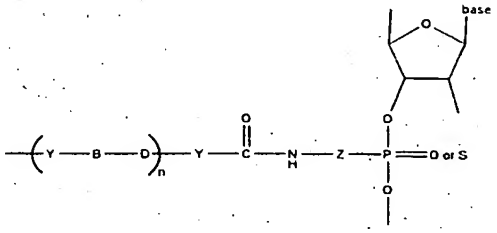


As will be appreciated by those in the art, Structure 20 has the terminal bond fixed as an amide bond.

In a preferred embodiment, a heteroatom linkage is used, i.e. oxo, amine, sulfur, etc. A preferred embodiment utilizes an amine linkage. Again, as outlined above for the amide linkages, for amine

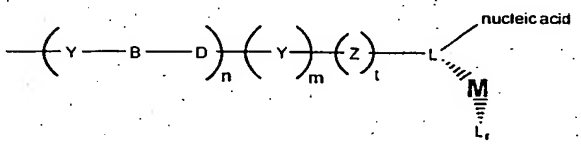
Structure 24 depicts a preferred embodiment, wherein the terminal B-D bond is an amide bond, the terminal Y is not present, and Z is a linker, as defined herein.

Structure 24

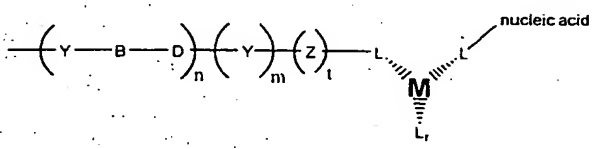


In a preferred embodiment, the conductive oligomer is covalently attached to the nucleic acid via a transition metal ligand. In this embodiment, the conductive oligomer is covalently attached to a ligand which provides one or more of the coordination atoms for a transition metal. In one embodiment, the ligand to which the conductive oligomer is attached also has the nucleic acid attached, as is generally depicted below in Structure 25. Alternatively, the conductive oligomer is attached to one ligand, and the nucleic acid is attached to another ligand, as is generally depicted below in Structure 26. Thus, in the presence of the transition metal, the conductive oligomer is covalently attached to the nucleic acid. Both of these structures depict Structure 3 conductive oligomers, although other oligomers may be utilized. Structures 25 and 26 depict two representative structures for nucleic acids; as will be appreciated by those in the art, it is possible to connect other types of capture binding ligands, for example proteinaceous binding ligands, in a similar manner:

Structure 25



Structure 26



In the structures depicted herein, M is a metal atom, with transition metals being preferred. Suitable transition metals for use in the invention include, but are not limited to, cadmium (Cd), copper (Cu), cobalt (Co), palladium (Pd), zinc (Zn), iron (Fe), ruthenium (Ru), rhodium (Rh), osmium (Os), rhenium (Re), platinium (Pt), scandium (Sc), titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), Molybdenum (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series of transition metals, the platinum metals (Ru, Rh, Pd, Os, Ir and Pt), along with Fe, Re, W, Mo and Tc, are preferred. Particularly preferred are ruthenium, rhenium, osmium, platinium, cobalt and iron.

The oxygen, sulfur, phosphorus and nitrogen-donating ligands are attached in such a manner as to allow the heteroatoms to serve as coordination atoms.

In a preferred embodiment, organometallic ligands are used. In addition to purely organic compounds for use as redox moieties, and various transition metal coordination complexes with δ-bonded organic ligand with donor atoms as heterocyclic or exocyclic substituents, there is available a wide variety of transition metal organometallic compounds with π-bonded organic ligands (see Advanced Inorganic Chemistry, 5th Ed., Cotton & Wilkinson, John Wiley & Sons, 1988, chapter 26; Organometallics, A Concise Introduction, Elschenbroich et al., 2nd Ed., 1992, VCH; and Comprehensive Organometallic Chemistry II, A Review of the Literature 1982-1994, Abel et al. Ed., Vol. 7, chapters 7, 8, 10 & 11, Pergamon Press, hereby expressly incorporated by reference). Such organometallic ligands include cyclic aromatic compounds such as the cyclopentadienide ion [$C_5H_5(-1)$] and various ring substituted and ring fused derivatives, such as the indenylide (-1) ion, that yield a class of bis(cyclopentadieyl) metal compounds, (i.e. the metallocenes); see for example Robins et al., J. Am. Chem. Soc. 104:1882-1893 (1982); and Gassman et al., J. Am. Chem. Soc. 108:4228-4229 (1986), incorporated by reference. Of these, ferrocene [$(C_5H_5)_2Fe$] and its derivatives are prototypical examples which have been used in a wide variety of chemical (Connelly et al., Chem. Rev. 96:877-910 (1996), incorporated by reference) and electrochemical (Geiger et al., Advances in Organometallic Chemistry 23:1-93; and Geiger et al., Advances in Organometallic Chemistry 24:87, incorporated by reference) electron transfer or "redox" reactions. Metallocene derivatives of a variety of the first, second and third row transition metals are potential candidates as redox moieties that are covalently attached to either the ribose ring or the nucleoside base of nucleic acid. Other potentially suitable organometallic ligands include cyclic arenes such as benzene, to yield bis(arene)metal compounds and their ring substituted and ring fused derivatives, of which bis(benzene)chromium is a prototypical example. Other acyclic π-bonded ligands such as the allyl(-1) ion, or butadiene yield potentially suitable organometallic compounds, and all such ligands, in conjuction with other π-bonded and δ-bonded ligands constitute the general class of organometallic compounds in which there is a metal to carbon bond. Electrochemical studies of various dimers and oligomers of such compounds with bridging organic ligands, and additional non-bridging ligands, as well as with and without metal-metal bonds are potential candidate redox moieties in nucleic acid analysis.

When one or more of the co-ligands is an organometallic ligand, the ligand is generally attached via one of the carbon atoms of the organometallic ligand, although attachment may be via other atoms for heterocyclic ligands. Preferred organometallic ligands include metallocene ligands, including substituted derivatives and the metalloceneophanes (see page 1174 of Cotton and Wilkenson, supra). For example, derivatives of metallocene ligands such as methylcyclopentadienyl, with multiple methyl groups being preferred, such as pentamethylcyclopentadienyl, can be used to increase the stability of

The use of metal ions to connect the binding ligands such as nucleic acids can serve as an internal control or calibration of the system, to evaluate the number of available binding ligands on the surface. However, as will be appreciated by those in the art, if metal ions are used to connect the binding ligands such as nucleic acids to the conductive oligomers, it is generally desirable to have this metal ion complex have a different redox potential than that of the ETMs used in the rest of the system, as described below. This is generally true so as to be able to distinguish the presence of the capture probe from the presence of the target analyte. This may be useful for identification, calibration and/or quantification. Thus, the amount of capture probe on an electrode may be compared to the amount of target analyte to quantify the amount of target sequence in a sample. This is quite significant to serve as an internal control of the sensor or system. This allows a measurement either prior to the addition of target or after, on the same molecules that will be used for detection, rather than rely on a similar but different control system. Thus, the actual molecules that will be used for the detection can be quantified prior to any experiment. This is a significant advantage over prior methods.

In a preferred embodiment, the capture binding ligands are covalently attached to the electrode via an insulator. The attachment of a variety of binding ligands such as proteins and nucleic acids to insulators such as alkyl groups is well known, and can be done to the nucleic acid bases or the backbone, including the ribose or phosphate for backbones containing these moieties, or to alternate backbones for nucleic acid analogs, or to the side chains or backbone of the amino acids.

In a preferred embodiment, there may be one or more different capture binding ligand species (sometimes referred to herein as "anchor ligands", "anchor probes" or "capture probes" with the phrase "probe" generally referring to nucleic acid species) on the surface, as is generally depicted in the Figures. In some embodiments, there may be one type of capture binding ligand, or one type of capture binding ligand extender, as is more fully described below. Alternatively, different capture binding ligands, or one capture binding ligand with a multiplicity of different capture extender binding ligands can be used. Similarly, when nucleic acid systems are used, it may be desirable to use auxillary capture probes that comprise relatively short probe sequences, that can be used to "tack down" components of the system, for example the recruitment linkers, to increase the concentration of ETMs at the surface.

Thus the present invention provides electrodes comprising monolayers comprising conductive oligomers and capture binding ligands, useful in target analyte detection systems.

In a preferred embodiment, the compositions further comprise a solution binding ligand. Solution binding ligands are similar to capture binding ligands, in that they bind to target analytes. The solution

In one embodiment, the electron donors and acceptors are redox proteins as are known in the art. However, redox proteins in many embodiments are not preferred.

The choice of the specific ETMs will be influenced by the type of electron transfer detection used, as is generally outlined below. Preferred ETMs are metallocenes, with ferrocene being particularly preferred.

In a preferred embodiment, a plurality of ETMs are used. As is shown in the examples, the use of multiple ETMs provides signal amplification and thus allows more sensitive detection limits. Accordingly, pluralities of ETMs are preferred, with at least about 2 ETMs per recruitment linker being preferred, and at least about 10 being particularly preferred, and at least about 20 to 50 being especially preferred. In some instances, very large numbers of ETMs (100 to 1000) can be used.

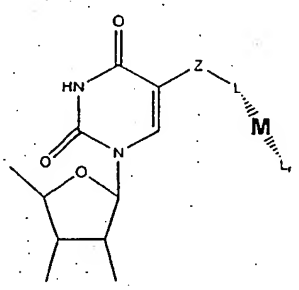
As will be appreciated by those in the art, the portion of the label probe (or target, in some embodiments) that comprises the ETMs (termed herein a "recruitment linker" or "signal carrier") can be nucleic acid, or it can be a non-nucleic acid linker that links the solution binding ligand to the ETMs. Thus, as will be appreciated by those in the art, there are a variety of configurations that can be used. In a preferred embodiment, the recruitment linker is nucleic acid (including analogs), and attachment of the ETMs can be via (1) a base; (2) the backbone, including the ribose, the phosphate, or comparable structures in nucleic acid analogs; (3) nucleoside replacement, described below; or (4) metallocene polymers, as described below. In a preferred embodiment, the recruitment linker is non-nucleic acid, and can be either a metallocene polymer or an alkyl-type polymer (including heteroalkyl, as is more fully described below) containing ETM substitution groups. These options are generally depicted in Figure 44.

In a preferred embodiment, the recruitment linker is a nucleic acid, and comprises covalently attached ETMs. The ETMs may be attached to nucleosides within the nucleic acid in a variety of positions. Preferred embodiments include, but are not limited to, (1) attachment to the base of the nucleoside, (2) attachment of the ETM as a base replacement, (3) attachment to the backbone of the nucleic acid, including either to a ribose of the ribose-phosphate backbone or to a phosphate moiety, or to analogous structures in nucleic acid analogs, and (4) attachment via metallocene polymers, with the latter being preferred.

In addition, as is described below, when the recruitment linker is nucleic acid, it may be desirable to use secondary label probes, that have a first portion that will hybridize to a portion of the primary label probes and a second portion comprising a recruitment linker as is defined herein. This is generally

Similarly, as for the conductive oligomers, the linkage may be done using a linker, which may utilize an amide linkage (see generally Telser et al., J. Am. Chem. Soc. 111:7221-7226 (1989); Telser et al., J. Am. Chem. Soc. 111:7226-7232 (1989), both of which are expressly incorporated by reference). These structures are generally depicted below in Structure 31, which again uses uridine as the base, although as above, the other bases may also be used:

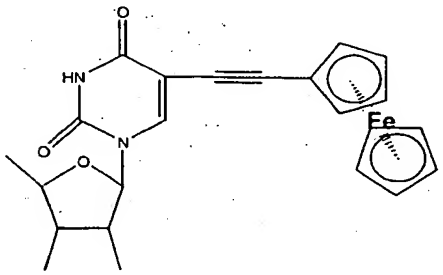
Structure 31



In this embodiment, L is a ligand as defined above, with L_r and M as defined above as well. Preferably, L is amino, phen, byp and terpy.

In a preferred embodiment, the ETM attached to a nucleoside is a metallocene; i.e. the L and L_r of Structure 31 are both metallocene ligands, L_m, as described above. Structure 32 depicts a preferred embodiment wherein the metallocene is ferrocene, and the base is uridine, although other bases may be used:

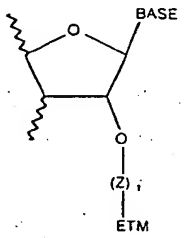
Structure 32



Preliminary data suggest that Structure 32 may cyclize, with the second acetylene carbon atom attacking the carbonyl oxygen, forming a furan-like structure. Preferred metallocenes include ferrocene, cobaltocene and osmiumocene.

In a preferred embodiment, the ETM is attached to a ribose at any position of the ribose-phosphate backbone of the nucleic acid, i.e. either the 5' or 3' terminus or any internal nucleoside. Ribose in this case can include ribose analogs. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose can be made, with nitrogen, oxygen, sulfur and phosphorus-containing modifications possible. Amino-modified and oxygen-modified ribose is preferred. See generally PCT

Structure 35

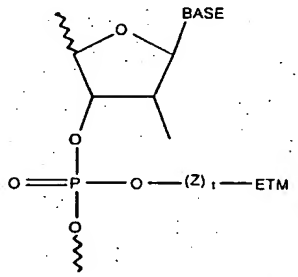


In Structure 35, Z is a linker, as defined herein, and t is either one or zero. Preferred Z linkers include alkyl groups including heteroalkyl groups such as $(CH_2)n$ and $(CH_2CH_2O)n$, with n from 1 to 10 being preferred, and n = 1 to 4 being especially preferred, and n=4 being particularly preferred.

Linkages utilizing other heteroatoms are also possible.

In a preferred embodiment, an ETM is attached to a phosphate at any position of the ribose-phosphate backbone of the nucleic acid. This may be done in a variety of ways. In one embodiment, phosphodiester bond analogs such as phosphoramide or phosphoramidite linkages may be incorporated into a nucleic acid, where the heteroatom (i.e. nitrogen) serves as a transition metal ligand (see PCT publication WO 95/15971, incorporated by reference). Alternatively, the conductive oligomers depicted in Structures 23 and 24 may be replaced by ETMs. In a preferred embodiment, the composition has the structure shown in Structure 36.

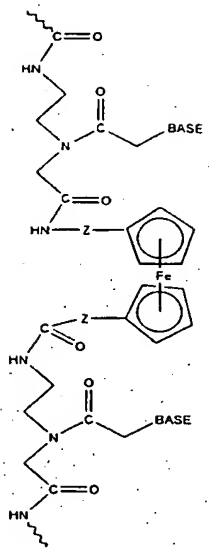
Structure 36



In Structure 361, the ETM is attached via a phosphate linkage, generally through the use of a linker, Z. Preferred Z linkers include alkyl groups, including heteroalkyl groups such as $(CH_2)_n$, $(CH_2CH_2O)_n$, with n from 1 to 10 being preferred, and n = 1 to 4 being especially preferred, and n=4 being particularly preferred.

When the ETM is attached to the base or the backbone of the nucleoside, it is possible to attach the ETMs via "dendrimer" structures, as is more fully outlined below. As is generally depicted in Figure 37, alkyl-based linkers can be used to create multiple branching structures comprising one or more ETMs at the terminus of each branch. Generally, this is done by creating branch points containing

Structure 38



In Structure 38, preferred Z groups are as listed above, and again, each Z linker can be the same or different. As above, other nucleic acid analogs may be used as well.

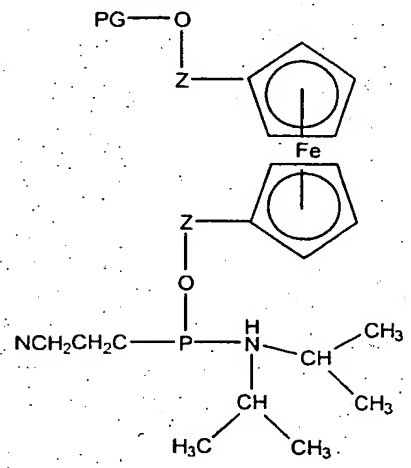
In addition, although the structures and discussion above depicts metallocenes, and particularly ferrocene, this same general idea can be used to add ETMs in addition to metallocenes, as nucleoside replacements or in polymer embodiments, described below. Thus, for example, when the ETM is a transition metal complex other than a metallocene, comprising one, two or three (or more) ligands, the ligands can be functionalized as depicted for the ferrocene to allow the addition of phosphoramidite groups. Particularly preferred in this embodiment are complexes comprising at least two ring (for example, aryl and substituted aryl) ligands, where each of the ligands comprises functional groups for attachment via phosphoramidite chemistry. As will be appreciated by those in the art, this type of reaction, creating polymers of ETMs either as a portion of the backbone of the nucleic acid or as "side groups" of the nucleic acids, to allow amplification of the signals generated herein, can be done with virtually any ETM that can be functionalized to contain the correct chemical groups.

Thus, by inserting a metallocene such as ferrocene (or other ETM) into the backbone of a nucleic acid, nucleic acid analogs are made; that is, the invention provides nucleic acids having a backbone comprising at least one metallocene. This is distinguished from nucleic acids having metallocenes attached to the backbone, i.e. via a ribose, a phosphate, etc. That is, two nucleic acids each made up of a traditional nucleic acid or analog (nucleic acids in this case including a single nucleoside), may be covalently attached to each other via a metallocene. Viewed differently, a metallocene derivative or substituted metallocene is provided, wherein each of the two aromatic rings of the metallocene has a nucleic acid substitutent group.

In Structure 39, PG is a protecting group, generally suitable for use in nucleic acid synthesis, with DMT, MMT and TMT all being preferred. The aromatic rings can either be the rings of the metallocene, or aromatic rings of ligands for transition metal complexes or other organic ETMs. The aromatic rings may be the same or different, and may be substituted as discussed herein.

Structure 40 depicts the ferrocene derivative:

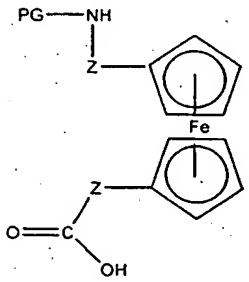
Structure 40



These phosphoramidite analogs can be added to standard oligonucleotide syntheses as is known in the art.

Structure 41 depicts the ferrocene peptide nucleic acid (PNA) monomer, that can be added to PNA synthesis (or regular protein synthesis) as is known in the art and depicted within the Figures and Examples:

Structure 41



In Structure 41, the PG protecting group is suitable for use in peptide nucleic acid synthesis, with MMT, boc and Fmoc being preferred.

These same intermediate compounds can be used to form ETM or metallocene polymers, which are added to the nucleic acids, rather than as backbone replacements, as is more fully described below.

In a preferred embodiment, the recruitment linker is not nucleic acid, and instead may be any sort of linker or polymer. As will be appreciated by those in the art, generally any linker or polymer that can be modified to contain ETMs can be used. In general, the polymers or linkers should be reasonably soluble and contain suitable functional groups for the addition of ETMs.

As used herein, a "recruitment polymer" comprises at least two or three subunits, which are covalently attached. At least some portion of the monomeric subunits contain functional groups for the covalent attachment of ETMs. In some embodiments coupling moieties are used to covalently link the subunits with the ETMs. Preferred functional groups for attachment are amino groups, carboxy groups, oxo groups and thiol groups, with amino groups being particularly preferred. As will be appreciated by those in the art, a wide variety of recruitment polymers are possible.

Suitable linkers include, but are not limited to, alkyl linkers (including heteroalkyl (including (poly)ethylene glycol-type structures), substituted alkyl, aryalkyl linkers, etc. As above for the polymers, the linkers will comprise one or more functional groups for the attachment of ETMs, which will be done as will be appreciated by those in the art, for example through the use homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference).

Suitable recruitment polymers include, but are not limited to, functionalized styrenes, such as amino styrene, functionalized dextrans, and polyamino acids. Preferred polymers are polyamino acids (both poly-D-amino acids and poly-L-amino acids), such as polylysine, and polymers containing lysine and other amino acids being particularly preferred. Other suitable polyamino acids are polyglutamic acid, polyaspartic acid, co-polymers of lysine and glutamic or aspartic acid, co-polymers of lysine with alanine, tyrosine, phenylalanine, serine, tryptophan, and/or proline.

In a preferred embodiment, the recruitment linker comprises a metallocene polymer, as is described above.

The attachment of the recruitment linkers to either the solution binding ligand or the first portion of the label probe will depend on the composition of the recruitment linker and of the label and/or binding ligand, as will be appreciated by those in the art. When either the label probe or the binding ligand is nucleic acid, nucleic acid recruitment linkers are generally formed during the synthesis of the first species, with incorporation of nucleosides containing ETMs as required. Alternatively, the first portion of the label probe or the binding ligand and the recruitment linker may be made separately, and then attached. When they are both nucleic acid, there may be an overlapping section of complementarity,

about 3 being preferred, or alternatively the ETMs should be spaced sufficiently far apart that the intervening nucleotides can sufficiently hybridize to allow good kinetics.

In one embodiment, when nucleic acid targets and/or binding ligands and/or recruitment linkers are used, non-covalently attached ETMs may be used. In one embodiment, the ETM is a hybridization indicator. Hybridization indicators serve as an ETM that will preferentially associate with double stranded nucleic acid is added, usually reversibly, similar to the method of Millan et al., Anal. Chem. 65:2317-2323 (1993); Millan et al., Anal. Chem. 662943-2948 (1994), both of which are hereby expressly incorporated by reference. In this embodiment, increases in the local concentration of ETMs, due to the association of the ETM hybridization indicator with double stranded nucleic acid at the surface, can be monitored using the monolayers comprising the conductive oligomers. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only occurs in the presence of double stranded nucleic acid, only in the presence of double stranded nucleic acid will the ETMs concentrate. Intercalating transition metal complex ETMs are known in the art. Similarly, major or minor groove binding moieties, such as methylene blue, may also be used in this embodiment.

Similarly, the systems of the invention may utilize non-covalently attached ETMs, as is generally described in Napier et al., Bioconj. Chem. 8:906 (1997), hereby expressly incorporated by reference. In this embodiment, changes in the redox state of certain molecules as a result of the presence of DNA (i.e. guanine oxidation by ruthenium complexes) can be detected using the SAMs comprising conductive oligomers as well.

Thus, the present invention provides electrodes comprising monolayers comprising conductive oligomers, generally including capture binding ligands, and either binding ligands or label probes that will bind to the binding ligands comprising recruitment linkers containing ETMs.

In a preferred embodiment, the compositions of the invention are used to detect target analytes in a sample. In a preferred embodiment, the target analyte is a nucleic acid, and thus detection of target sequences is done. The term "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in

Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al, hereby incorporated by referenece. The hybridization conditions may also vary when a non-ionic backbone, i.e. PNA is used, as is known in the art. In addition, cross-linking agents may be added after target binding to cross-link, i.e. covalently attach, the two strands of the hybridization complex.

As will be appreciated by those in the art, the nucleic acid systems of the invention may take on a large number of different configurations, as is generally depicted in the figures. In general, there are three types of systems that can be used: (1) systems in which the target analyte itself is labeled with ETMs (i.e. the use of a target analyte analog, for non-nucleic acid systems, or, for nucleic acid systems, the target sequence is labeled; see Figures 6A, 6B and 6C); (2) systems in which label probes (or capture binding ligands with recruitment linkers) directly bind (i.e. hybridize for nucleic acids) to the target analytes (see Figures 6D-6H for nucleic acid embodiments and Figure 2A and 2C for non-nucleic acid embodiments); and (3) systems in which label probes comprising recruitment linkers are indirectly bound to the target analytes, for example through the use of amplifier probes (see Figures 6I, 6J and 6K for nucleic acid embodiments and Figure 2B and 2D for non-nucleic acid embodiments).

In all three of these systems, it is preferred, although not required, that the target analyte be immobilized on the electrode surface. This is preferably done using capture binding ligands and optionally one or more capture extender ligands. When only capture binding ligands are utilized, it is necessary to have unique capture binding ligands for each target analyte; that is, the surface must be customized to contain unique capture binding ligands. Alternatively, the use of capture extender ligands, particularly when the capture extender ligands are capture extender probes (i.e. nucleic acids) may be used, that allow a "universal" surface, i.e. a surface containing a single type of capture probe that can be used to detect any target sequence.

Capture extender probes or moieties may take on a variety of different conformations, depending on the identity of the target analyte and of the binding ligands. In a preferred embodiment, the target analyte and the binding ligand are nucleic acids. In this embodiment, the "capture extender" probes are generally depicted in Figure 6 and have a first portion that will hybridize to all or part of the capture probe, and a second portion that will hybridize to a portion of the target sequence. This then allows the generation of customized soluble probes, which as will be appreciated by those in the art is generally simpler and less costly. As shown herein (e.g. Figure 6H), two capture extender probes may be used. This has generally been done to stabilize assay complexes (for example when the target sequence is large, or when large amplifier probes (particularly branched or dendrimer amplifier probes) are used.

In one embodiment, the target is not bound to the electrode surface using capture binding ligands. In this embodiment, what is important, as for all the assays herein, is that excess label probes be removed prior to detection and that the assay complex (comprising the recruitment linker) be in proximity to the surface. As will be appreciated by those in the art, this may be accomplished in other ways. For example, the assay complex may be present on beads that are added to the electrode comprising the monolayer. The recruitment linkers comprising the ETMs may be placed in proximity to the conductive oligomer surface using techniques well known in the art, including gravity settling of the beads on the surface, electrostatic or magnetic interactions between bead components and the surface, using binding partner attachment as outlined above. Alternatively, after the removal of excess reagents such as excess label probes, the assay complex may be driven down to the surface, for example by pulsing the system with a voltage sufficient to drive the assay complex to the surface.

However, preferred embodiments utilize assay complexes attached via capture binding ligands.

For nucleic acid systems, a preferred embodiments utilize the target sequence itself containing the ETMs. As discussed above, this may be done using target sequences that have ETMs incorporated at any number of positions, as outlined above. Representative examples are depicted in Figures 6A, 6B and 6C. In this embodiment, as for the others of the system, the 3'-5' orientation of the probes and targets is chosen to get the ETM-containing structures (i.e. recruitment linkers or target sequences) as close to the surface of the monolayer as possible, and in the correct orientation. This may be done using attachment via insulators or conductive oligomers as is generally shown in the Figures. In addition, as will be appreciated by those in the art, multiple capture probes can be utilized, either in a configuration such as depicted in Figure 6C, wherein the 5'-3' orientation of the capture probes is different, or where "loops" of target form when multiples of capture probes as depicted in Figures 6A and 6B are used.

For nucleic acid systems, a preferred embodiments utilize the label probes directly hybridizing to the target sequences, as is generally depicted in Figures 6D - 6I. In these embodiments, the target sequence is preferably, but not required to be, immobilized on the surface using capture probes, including capture extender probes. Label probes are then used to bring the ETMs into proximity of the surface of the monolayer comprising conductive oligomers. In a preferred embodiment, multiple label probes are used; that is, label probes are designed such that the portion that hybridizes to the target sequence (labeled **41** in the figures) can be different for a number of different label probes, such that amplification of the signal occurs, since multiple label probes can bind for every target sequence. Thus, as depicted in the figures, n is an integer of at least one. Depending on the sensitivity desired, the length of the target sequence, the number of ETMs per label probe, etc., preferred ranges of n are from 1 to 50, with from about 1 to about 20 being particularly preferred, and from about 2 to about 5

ligands comprising recruitment linkers. However, amplifier probes are preferred in nucleic acid systems.

Amplifier probes comprise a first probe sequence that is used, either directly or indirectly, to hybridize to the target sequence. That is, the amplifier probe itself may have a first probe sequence that is substantially complementary to the target sequence (e.g. Figure 6I), or it has a first probe sequence that is substantially complementary to a portion of an additional probe, in this case called a label extender probe, that has a first portion that is substantially complementary to the target sequence (e.g. Figure 6N). In a preferred embodiment, the first probe sequence of the amplifier probe is substantially complementary to the target sequence, as is generally depicted in Figure 6I.

In general, as for all the probes herein, the first probe sequence is of a length sufficient to give specificity and stability. Thus generally, the probe sequences of the invention that are designed to hybridize to another nucleic acid (i.e. probe sequences, amplification sequences, portions or domains of larger probes) are at least about 5 nucleosides long, with at least about 10 being preferred and at least about 15 being especially preferred.

In a preferred embodiment, as is depicted in Figure 8, the amplifier probes, or any of the other probes of the invention, may form hairpin stem-loop structures in the absence of their target. The length of the stem double-stranded sequence will be selected such that the hairpin structure is not favored in the presence of target. The use of these type of probes, in the systems of the invention or in any nucleic acid detection systems, can result in a significant decrease in non-specific binding and thus an increase in the signal to noise ratio.

Generally, these hairpin structures comprise four components. The first component is a target binding sequence, i.e. a region complementary to the target (which may be the sample target sequence or another probe sequence to which binding is desired), that is about 10 nucleosides long, with about 15 being preferred. The second component is a loop sequence, that can facilitate the formation of nucleic acid loops. Particularly preferred in this regard are repeats of GTC, which has been identified in Fragile X Syndrome as forming turns. (When PNA analogs are used, turns comprising proline residues may be preferred). Generally, from three to five repeats are used, with four to five being preferred. The third component is a self-complementary region, which has a first portion that is complementary to a portion of the target sequence region and a second portion that comprises a first portion of the label probe binding sequence. The fourth component is substantially complementary to a label probe (or other probe, as the case may be). The fourth component further comprises a "sticky end", that is, a portion that does not hybridize to any other portion of the probe, and preferably contains most, if not all, of the ETMs. The general structure is depicted in Figure 38. As will be

In a preferred embodiment, more than one label extender probe is used with a single amplifier probe to reduce non-specific binding, as is depicted in Figure 6O and generally outlined in U.S. Patent No. 5,681,697, incorporated by reference herein. In this embodiment, a first portion of the first label extender probe hybridizes to a first portion of the target sequence, and the second portion of the first label extender probe hybridizes to a first probe sequence of the amplifier probe. A first portion of the second label extender probe hybridizes to a second portion of the target sequence, and the second portion of the second label extender probe hybridizes to a second probe sequence of the amplifier probe. These form structures sometimes referred to as "cruciform" structures or configurations, and are generally done to confer stability when large branched or dendrimeric amplifier probes are used.

In addition, as will be appreciated by those in the art, the label extender probes may interact with a preamplifier probe, described below, rather than the amplifier probe directly.

Similarly, as outlined above, a preferred embodiment utilizes several different amplifier probes, each with first probe sequences that will hybridize to a different portion of the label extender probe. In addition, as outlined above, it is also possible that the different amplifier probes contain different amplification sequences, although this is generally not preferred.

In addition to the first probe sequence, the amplifier probe also comprises at least one amplification sequence. An "amplification sequence" or "amplification segment" or grammatical equivalents herein is meant a sequence that is used, either directly or indirectly, to bind to a first portion of a label probe as is more fully described below. Preferably, the amplifier probe comprises a multiplicity of amplification sequences, with from about 3 to about 1000 being preferred, from about 10 to about 100 being particularly preferred, and about 50 being especially preferred. In some cases, for example when linear amplifier probes are used, from 1 to about 20 is preferred with from about 5 to about 10 being particularly preferred. Again, when non-nucleic acid amplifier moieties are used, the amplification segment can bind label ligands.

The amplification sequences may be linked to each other in a variety of ways, as will be appreciated by those in the art. They may be covalently linked directly to each other, or to intervening sequences or chemical moieties, through nucleic acid linkages such as phosphodiester bonds, PNA bonds, etc., or through interposed linking agents such amino acid, carbohydrate or polyol bridges, or through other cross-linking agents or binding partners. The site(s) of linkage may be at the ends of a segment, and/or at one or more internal nucleotides in the strand. In a preferred embodiment, the amplification sequences are attached via nucleic acid linkages.

amplifier probe are substantially complementary to a first portion of a label probe. Alternatively, amplifier extender probes are used, that have a first portion that binds to the amplification sequence and a second portion that binds to the first portion of the label probe.

In addition, the compositions of the invention may include "preamplifier" molecules, which serves a bridging moiety between the label extender molecules and the amplifier probes. In this way, more amplifier and thus more ETMs are ultimately bound to the detection probes. Preamplifier molecules may be either linear or branched, and typically contain in the range of about 30-3000 nucleotides.

The reactions outlined below may be accomplished in a variety of ways, as will be appreciated by those in the art. Components of the reaction may be added simultaneously, or sequentially, in any order, with preferred embodiments outlined below. In addition, the reaction may include a variety of other reagents may be included in the assays. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used, depending on the sample preparation methods and purity of the target.

Generally, the methods are as follows. In a preferred embodiment, the target is initially immobilized or attached to the electrode. For nucleic acids, this is done by forming a hybridization complex between a capture probe and a portion of the target sequence. A preferred embodiment utilizes capture extender probes; in this embodiment, a hybridization complex is formed between a portion of the target sequence and a first portion of a capture extender probe, and an additional hybridization complex between a second portion of the capture extender probe and a portion of the capture probe. Additional preferred embodiments utilize additional capture probes, thus forming a hybridization complex between a portion of the target sequence and a first portion of a second capture extender probe, and an additional hybridization complex between a second portion of the second capture extender probe and a second portion of the capture probe. Non-nucleic acid embodiments utilize capture binding ligands and optional capture extender ligands.

Alternatively, the attachment of the target sequence to the electrode is done simultaneously with the other reactions.

The method proceeds with the introduction of amplifier probes, if utilized. In a preferred embodiment, the amplifier probe comprises a first probe sequence that is substantially complementary to a portion of the target sequence, and at least one amplification sequence.

In a preferred embodiment, the first probe sequence of the amplifier probe is hybridized to a first portion of at least one label extender probe, and a second portion of the label extender probe is hybridized to a portion of the target sequence. Other preferred embodiments utilize more than one label extender probe, as is generally shown in Figure 6O.

In a preferred embodiment, the amplification sequences of the amplifier probe are used directly for detection, by hybridizing at least one label probe sequence.

The invention thus provides assay complexes that minimally comprise a target sequence and a label probe. "Assay complex" herein is meant the collection of binding complexes comprising capture binding ligands, target analytes (or analogs, as described below) and label moieties comprising recruitment linkers that allows detection. The composition of the assay complex depends on the use of the different components outlined herein. Thus, in Figure 6A, the assay complex comprises the capture probe and the target sequence. The assay complexes may also include capture extender ligands (including probes), label extender ligands, and amplifier ligands, as outlined herein, depending on the configuration used.

The assays are generally run under conditions which allows formation of the assay complex only in the presence of target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration pH, organic solvent concentration, etc.

These parameters may also be used to control non-specific binding for nucleic acids, as is generally outlined in U.S. Patent No. 5,681,697. Thus it may be desirable to perform certain steps at higher stringency conditions; for example, when an initial hybridization step is done between the target sequence and the label extender and capture extender probes. Running this step at conditions which favor specific binding can allow the reduction of non-specific binding.

In a preferred embodiment, when all of the components outlined herein are used, a preferred method for nucleic acid detection is as follows. Single-stranded target sequence is incubated under hybridization conditions with the capture extender probes and the label extender probes. A preferred embodiment does this reaction in the presence of the electrode with immobilized capture probes, although this may also be done in two steps, with the initial incubation and the subsequent addition to the electrode. Excess reagents are washed off, and amplifier probes are then added. If preamplifier probes are used, they may be added either prior to the amplifier probes or simultaneously with the amplifier probes. Excess reagents are washed off, and label probes are then added. Excess reagents are washed off, and detection proceeds as outlined below.

either ribose or phosphate in the backbone. In these embodiments, for attachment at positions other than the base, attachment is done as will be appreciated by those in the art, depending on the backbone. Thus, for example, attachment can be made at the carbon atoms of the PNA backbone, as is described below, or at either terminus of the PNA.

The compositions may be made in several ways. A preferred method first synthesizes a conductive oligomer attached to a nucleoside, with addition of additional nucleosides to form the capture probe followed by attachment to the electrode. Alternatively, the whole capture probe may be made and then the completed conductive oligomer added, followed by attachment to the electrode. Alternatively, a monolayer of conductive oligomer (some of which have functional groups for attachment of capture probes) is attached to the electrode first, followed by attachment of the capture probe. The latter two methods may be preferred when conductive oligomers are used which are not stable in the solvents and under the conditions used in traditional nucleic acid synthesis.

In a preferred embodiment, the compositions of the invention are made by first forming the conductive oligomer covalently attached to the nucleoside, followed by the addition of additional nucleosides to form a capture probe nucleic acid, with the last step comprising the addition of the conductive oligomer to the electrode.

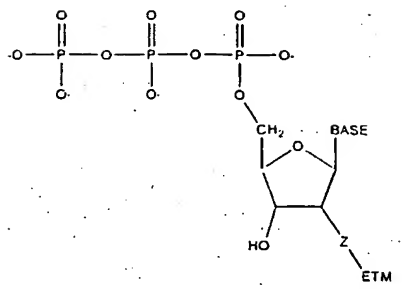
The attachment of the conductive oligomer to the nucleoside may be done in several ways. In a preferred embodiment, all or part of the conductive oligomer is synthesized first (generally with a functional group on the end for attachment to the electrode), which is then attached to the nucleoside. Additional nucleosides are then added as required, with the last step generally being attachment to the electrode. Alternatively, oligomer units are added one at a time to the nucleoside, with addition of additional nucleosides and attachment to the electrode. A number of representative syntheses are shown in the Figures of WO 98/20162, PCT US98/12430, PCT US99/01705 and PCT US99/01703, all of which are expressly incorporated by reference.

The conductive oligomer is then attached to a nucleoside that may contain one (or more) of the oligomer units, attached as depicted herein.

In a preferred embodiment, attachment is to a ribose of the ribose-phosphate backbone in a number of ways, including attachment via amide and amine linkages. In a preferred embodiment, there is at least a methylene group or other short aliphatic alkyl groups (as a Z group) between the nitrogen attached to the ribose and the aromatic ring of the conductive oligomer.

Alternatively, attachment is via a phosphate of the ribose-phosphate backbone.

Structure 43



Thus, in some embodiments, it may be possible to generate the nucleic acids comprising ETMs in situ. For example, a target sequence can hybridize to a capture probe (for example on the surface) in such a way that the terminus of the target sequence is exposed, i.e. unhybridized. The addition of enzyme and triphosphate nucleotides labelled with ETMs allows the in situ creation of the label. Similarly, using labeled nucleotides recognized by polymerases can allow simultaneous PCR and detection; that is, the target sequences are generated in situ.

In a preferred embodiment, the modified nucleoside is converted to the phosphoramidite or H-phosphonate form, which are then used in solid-phase or solution syntheses of oligonucleotides. In this way the modified nucleoside, either for attachment at the ribose (i.e. amino- or thiol-modified nucleosides) or the base, is incorporated into the oligonucleotide at either an internal position or the 5' terminus. This is generally done in one of two ways. First, the 5' position of the ribose is protected with 4',4-dimethoxytrityl (DMT) followed by reaction with either 2-cyanoethoxy-bis-diisopropylaminophosphine in the presence of diisopropylammonium tetrazolide, or by reaction with chlorodiisopropylamino 2'-cyanoethyoxyphosphine, to give the phosphoramidite as is known in the art; although other techniques may be used as will be appreciated by those in the art. See Gait, supra; Caruthers, Science 230:281 (1985), both of which are expressly incorporated herein by reference.

For attachment of a group to the 3' terminus, a preferred method utilizes the attachment of the modified nucleoside (or the nucleoside replacement) to controlled pore glass (CPG) or other oligomeric supports. In this embodiment, the modified nucleoside is protected at the 5' end with DMT, and then reacted with succinic anhydride with activation. The resulting succinyl compound is attached to CPG or other oligomeric supports as is known in the art. Further phosphoramidite nucleosides are added, either modified or not, to the 5' end after deprotection. Thus, the present invention provides conductive oligomers or insulators covalently attached to nucleosides attached to solid oligomeric supports such as CPG, and phosphoramidite derivatives of the nucleosides of the invention.

The invention further provides methods of making label probes with recruitment linkers comprising ETMs. These synthetic reactions will depend on the character of the recruitment linker and the

In one embodiment, the nucleosides are made with transition metal ligands, incorporated into a nucleic acid, and then the transition metal ion and any remaining necessary ligands are added as is known in the art. In an alternative embodiment, the transition metal ion and additional ligands are added prior to incorporation into the nucleic acid.

Once the nucleic acids of the invention are made, with a covalently attached attachment linker (i.e. either an insulator or a conductive oligomer), the attachment linker is attached to the electrode. The method will vary depending on the type of electrode used. As is described herein, the attachment linkers are generally made with a terminal "A" linker to facilitate attachment to the electrode. For the purposes of this application, a sulfur-gold attachment is considered a covalent attachment.

In a preferred embodiment, conductive oligomers, insulators, and attachment linkers are covalently attached via sulfur linkages to the electrode. However, surprisingly, traditional protecting groups for use of attaching molecules to gold electrodes are generally not ideal for use in both synthesis of the compositions described herein and inclusion in oligonucleotide synthetic reactions. Accordingly, the present invention provides novel methods for the attachment of conductive oligomers to gold electrodes, utilizing unusual protecting groups, including ethylpyridine, and trimethylsilylethyl as is depicted in the Figures. However, as will be appreciated by those in the art, when the conductive oligomers do not contain nucleic acids, traditional protecting groups such as acetyl groups and others may be used. See Greene et al., supra.

This may be done in several ways. In a preferred embodiment, the subunit of the conductive oligomer which contains the sulfur atom for attachment to the electrode is protected with an ethyl-pyridine or trimethylsilylethyl group. For the former, this is generally done by contacting the subunit containing the sulfur atom (preferably in the form of a sulfhydryl) with a vinyl pyridine group or vinyl trimethylsilylethyl group under conditions whereby an ethylpyridine group or trimethylsilylethyl group is added to the sulfur atom.

This subunit also generally contains a functional moiety for attachment of additional subunits, and thus additional subunits are attached to form the conductive oligomer. The conductive oligomer is then attached to a nucleoside, and additional nucleosides attached. The protecting group is then removed and the sulfur-gold covalent attachment is made. Alternatively, all or part of the conductive oligomer is made, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. The conductive oligomer is then attached to a nucleoside, and additional nucleosides attached. Alternatively, the conductive oligomer attached to a nucleic acid is made, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. Alternatively, the ethyl pyridine protecting group may be used as above, but removed after

In a preferred embodiment, the nucleic acid is a peptide nucleic acid or analog. In this embodiment, the invention provides peptide nucleic acids with at least one covalently attached ETM or attachment linker. In a preferred embodiment, these moieties are covalently attached to an monomeric subunit of the PNA. By "monomeric subunit of PNA" herein is meant the -NH-CH_2CH_2-N($COCH_2$-Base)-CH_2-CO- monomer, or derivatives (herein included within the definition of "nucleoside") of PNA. For example, the number of carbon atoms in the PNA backbone may be altered; see generally Nielsen et al., Chem. Soc. Rev. 1997 page 73, which discloses a number of PNA derivatives, herein expressly incorporated by reference. Similarly, the amide bond linking the base to the backbone may be altered; phosphoramide and sulfuramide bonds may be used. Alternatively, the moieties are attached to an internal monomeric subunit. By "internal" herein is meant that the monomeric subunit is not either the N-terminal monomeric subunit or the C-terminal monomeric subunit. In this embodiment, the moieties can be attached either to a base or to the backbone of the monomeric subunit. Attachment to the base is done as outlined herein or known in the literature. In general, the moieties are added to a base which is then incorporated into a PNA as outlined herein. The base may be either protected, as required for incorporation into the PNA synthetic reaction, or derivatized, to allow incorporation, either prior to the addition of the chemical substituent or afterwards. Protection and derivatization of the bases is shown in the Figures. The bases can then be incorporated into monomeric subunits; the figures depict two different chemical substituents, an ETM and a conductive oligomer, attached at a base.

In a preferred embodiment, the moieties are covalently attached to the backbone of the PNA monomer. The attachment is generally to one of the unsubstituted carbon atoms of the monomeric subunit, preferably the α-carbon of the backbone, as is depicted in the Figures, although attachment at either of the carbon 1 or 2 positions, or the α-carbon of the amide bond linking the base to the backbone may be done. In the case of PNA analogs, other carbons or atoms may be substituted as well. In a preferred embodiment, moieties are added at the α-carbon atoms, either to a terminal monomeric subunit or an internal one.

In this embodiment, a modified monomeric subunit is synthesized with an ETM or an attachment linker, or a functional group for its attachment, and then the base is added and the modified monomer can be incorporated into a growing PNA chain. The figures depict the synthesis of a conductive oligomer covalently attached to the backbone of a PNA monomeric subunit, and the synthesis of a ferrocene attached to the backbone of a monomeric subunit.

Once generated, the monomeric subunits with covalently attached moieties are incorporated into a PNA using the techniques outlined in Will et al., Tetrahedron 51(44):12069-12082 (1995), and Vanderlaan et al., Tett. Let. 38:2249-2252 (1997), both of which are hereby expressly incorporated in

concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

Candidate agents may be added either before or after the target analyte.

Once the assay complexes of the invention are made, that minimally comprise a target sequence and a label probe, detection proceeds with electronic initiation. Without being limited by the mechanism or theory, detection is based on the transfer of electrons from the ETM to the electrode.

Detection of electron transfer, i.e. the presence of the ETMs, is generally initiated electronically, with voltage being preferred. A potential is applied to the assay complex. Precise control and variations in the applied potential can be via a potentiostat and either a three electrode system (one reference, one sample (or working) and one counter electrode) or a two electrode system (one sample and one counter electrode). This allows matching of applied potential to peak potential of the system which depends in part on the choice of ETMs and in part on the conductive oligomer used, the composition and integrity of the monolayer, and what type of reference electrode is used. As described herein, ferrocene is a preferred ETM.

any electrons from the luminol electron source, since the voltages are less than the redox potential of the luminol. However, at or above the redox potential of luminol, the luminol then transfers an electron to the ETM, allowing rapid and repeated electron transfer. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the ETM of the label probe.

Luminol has the added benefit of becoming a chemiluminiscent species upon oxidation (see Jirka et al., Analytica Chimica Acta 284:345 (1993)), thus allowing photo-detection of electron transfer from the ETM to the electrode. Thus, as long as the luminol is unable to contact the electrode directly, i.e. in the presence of the SAM such that there is no efficient electron transfer pathway to the electrode, luminol can only be oxidized by transferring an electron to the ETM on the label probe. When the ETM is not present, i.e. when the target sequence is not hybridized to the composition of the invention, luminol is not significantly oxidized, resulting in a low photon emission and thus a low (if any) signal from the luminol. In the presence of the target, a much larger signal is generated. Thus, the measure of luminol oxidation by photon emission is an indirect measurement of the ability of the ETM to donate electrons to the electrode. Furthermore, since photon detection is generally more sensitive than electronic detection, the sensitivity of the system may be increased. Initial results suggest that luminescence may depend on hydrogen peroxide concentration, pH, and luminol concentration, the latter of which appears to be non-linear.

Suitable electron source molecules are well known in the art, and include, but are not limited to, ferricyanide, and luminol.

Alternatively, output electron acceptors or sinks could be used, i.e. the above reactions could be run in reverse, with the ETM such as a metallocene receiving an electron from the electrode, converting it to the metallicenium, with the output electron acceptor then accepting the electron rapidly and repeatedly. In this embodiment, cobalticenium is the preferred ETM.

The presence of the ETMs at the surface of the monolayer can be detected in a variety of ways. A variety of detection methods may be used, including, but not limited to, optical detection (as a result of spectral changes upon changes in redox states), which includes fluorescence, phosphorescence, luminiscence, chemiluminescence, electrochemiluminescence, and refractive index; and electronic detection, including, but not limited to, amperommetry, voltammetry, capacitance and impedence. These methods include time or frequency dependent methods based on AC or DC currents, pulsed methods, lock-in techniques, filtering (high pass, low pass, band pass), and time-resolved techniques including time-resolved fluoroscence.

Preferred examples display efficient fluorescence (reasonably high quantum yields) as well as low reorganization energies. These include Ru(4,7-biphenyl$_2$-phenanthroline)$_3^{2+}$, Ru(4,4'-diphenyl-2,2'-bipyridine)$_3^{2+}$ and platinum complexes (see Cummings et al., J. Am. Chem. Soc. 118:1949-1960 (1996), incorporated by reference). Alternatively, a *reduction* in fluorescence associated with hybridization can be measured using these systems.

In a further embodiment, electrochemiluminescence is used as the basis of the electron transfer detection. With some ETMs such as $Ru^{2+}(bpy)_3$, direct luminescence accompanies excited state decay. Changes in this property are associated with nucleic acid hybridization and can be monitored with a simple photomultiplier tube arrangement (see Blackburn, G. F. *Clin. Chem.* **37**: 1534-1539 (1991); and Juris et al., supra.

In a preferred embodiment, electronic detection is used, including amperommetry, voltammetry, capacitance, and impedence. Suitable techniques include, but are not limited to, electrogravimetry; coulometry (including controlled potential coulometry and constant current coulometry); voltametry (cyclic voltametry, pulse voltametry (normal pulse voltametry, square wave voltametry, differential pulse voltametry, Osteryoung square wave voltametry, and coulostatic pulse techniques); stripping analysis (aniodic stripping analysis, cathiodic stripping analysis, square wave stripping voltammetry); conductance measurements (electrolytic conductance, direct analysis); time-dependent electrochemical analyses (chronoamperometry, chronopotentiometry, cyclic chronopotentiometry and amperometry, AC polography, chronogalvametry, and chronocoulometry); AC impedance measurement; capacitance measurement; AC voltametry; and photoelectrochemistry.

In a preferred embodiment, monitoring electron transfer is via amperometric detection. This method of detection involves applying a potential (as compared to a separate reference electrode) between the nucleic acid-conjugated electrode and a reference (counter) electrode in the sample containing target genes of interest. Electron transfer of differing efficiencies is induced in samples in the presence or absence of target nucleic acid; that is, the presence or absence of the target nucleic acid, and thus the label probe, can result in different currents.

The device for measuring electron transfer amperometrically involves sensitive current detection and includes a means of controlling the voltage potential, usually a potentiostat. This voltage is optimized with reference to the potential of the electron donating complex on the label probe. Possible electron donating complexes include those previously mentioned with complexes of iron, osmium, platinum, cobalt, rhenium and ruthenium being preferred and complexes of iron being most preferred.

E_{DC} is the electrode potential, E_0 is the formal potential of the metal complex, R is the gas constant, T is the temperature in degrees Kelvin, n is the number of electrons transferred, F is faraday's constant, [O] is the concentration of oxidized molecules and [R] is the concentration of reduced molecules.

The Nernst equation can be rearranged as shown in Equations 2 and 3:

Equation 2

$$E_{DC} - E_0 = \frac{RT}{nF} \ln \frac{[O]}{[R]} \qquad (2)$$

E_{DC} is the DC component of the potential.

Equation 3

$$\exp^{\frac{nF}{RT}(E_{DC} - E_0)} = \frac{[O]}{[R]} \qquad (3)$$

Equation 3 can be rearranged as follows, using normalization of the concentration to equal 1 for simplicity, as shown in Equations 4, 5 and 6. This requires the subsequent multiplication by the total number of molecules.

Equation 4 $[O] + [R] = 1$

Equation 5 $[O] = 1 - [R]$

Equation 6 $[R] = 1 - [O]$

Plugging Equation 5 and 6 into Equation 3, and the fact that nF/RT equals 38.9 V^{-1}, for n=1, gives Equations 7 and 8, which define [O] and [R], respectively:

Equation 7

$$[O] = \frac{\exp^{38.9(E-E_0)}}{1 + \exp^{38.9(E-E_0)}} \qquad (4)$$

Equation 8

$$[R] = \frac{1}{1 + \exp^{38.9(E-E_0)}} \qquad (5)$$

Equation 12

$$i_{AC} = f(\text{Nernst factors})f(k_{ET})f(\text{instrument factors})$$

These equations can be used to model and predict the expected AC currents in systems which use input signals comprising both AC and DC components. As outlined above, traditional theory surprisingly does not model these systems at all, except for very low voltages.

In general, non-specifically bound label probes/ETMs show differences in impedance (i.e. higher impedances) than when the label probes containing the ETMs are specifically bound in the correct orientation. In a preferred embodiment, the non-specifically bound material is washed away, resulting in an effective impedance of infinity. Thus, AC detection gives several advantages as is generally discussed below, including an increase in sensitivity, and the ability to "filter out" background noise. In particular, changes in impedance (including, for example, bulk impedance) as between non-specific binding of ETM-containing probes and target-specific assay complex formation may be monitored.

Accordingly, when using AC initiation and detection methods, the frequency response of the system changes as a result of the presence of the ETM. By "frequency response" herein is meant a modification of signals as a result of electron transfer between the electrode and the ETM. This modification is different depending on signal frequency. A frequency response includes AC currents at one or more frequencies, phase shifts, DC offset voltages, faradaic impedance, etc.

Once the assay complex including the target sequence and label probe is made, a first input electrical signal is then applied to the system, preferably via at least the sample electrode (containing the complexes of the invention) and the counter electrode, to initiate electron transfer between the electrode and the ETM. Three electrode systems may also be used, with the voltage applied to the reference and working electrodes. The first input signal comprises at least an AC component. The AC component may be of variable amplitude and frequency. Generally, for use in the present methods, the AC amplitude ranges from about 1 mV to about 1.1 V, with from about 10 mV to about 800 mV being preferred, and from about 10 mV to about 500 mV being especially preferred. The AC frequency ranges from about 0.01 Hz to about 100 MHz, with from about 10 Hz to about 10 MHz being preferred, and from about 100 Hz to about 20 MHz being especially preferred.

The use of combinations of AC and DC signals gives a variety of advantages, including surprising sensitivity and signal maximization.

In a preferred embodiment, the first input signal comprises a DC component and an AC component. That is, a DC offset voltage between the sample and counter electrodes is swept through the

In a preferred embodiment, the AC frequency is varied. At different frequencies, different molecules respond in different ways. As will be appreciated by those in the art, increasing the frequency generally increases the output current. However, when the frequency is greater than the rate at which electrons may travel between the electrode and the ETM, higher frequencies result in a loss or decrease of output signal. At some point, the frequency will be greater than the rate of electron transfer between the ETM and the electrode, and then the output signal will also drop.

In one embodiment, detection utilizes a single measurement of output signal at a single frequency. That is, the frequency response of the system in the absence of target sequence, and thus the absence of label probe containing ETMs, can be previously determined to be very low at a particular high frequency. Using this information, any response at a particular frequency, will show the presence of the assay complex. That is, any response at a particular frequency is characteristic of the assay complex. Thus, it may only be necessary to use a single input high frequency, and any changes in frequency response is an indication that the ETM is present, and thus that the target sequence is present.

In addition, the use of AC techniques allows the significant reduction of background signals at any single frequency due to entities other than the ETMs, i.e. "locking out" or "filtering" unwanted signals. That is, the frequency response of a charge carrier or redox active molecule in solution will be limited by its diffusion coefficient and charge transfer coefficient. Accordingly, at high frequencies, a charge carrier may not diffuse rapidly enough to transfer its charge to the electrode, and/or the charge transfer kinetics may not be fast enough. This is particularly significant in embodiments that do not have good monolayers, i.e. have partial or insufficient monolayers, i.e. where the solvent is accessible to the electrode. As outlined above, in DC techniques, the presence of "holes" where the electrode is accessible to the solvent can result in solvent charge carriers "short circuiting" the system, i.e. the reach the electrode and generate background signal. However, using the present AC techniques, one or more frequencies can be chosen that prevent a frequency response of one or more charge carriers in solution, whether or not a monolayer is present. This is particularly significant since many biological fluids such as blood contain significant amounts of redox active molecules which can interfere with amperometric detection methods.

In a preferred embodiment, measurements of the system are taken at at least two separate frequencies, with measurements at a plurality of frequencies being preferred. A plurality of frequencies includes a scan. For example, measuring the output signal, e.g., the AC current, at a low input frequency such as 1 - 20 Hz, and comparing the response to the output signal at high frequency such as 10 - 100 kHz will show a frequency response difference between the presence and absence

Thus, the assay complexes comprising the nucleic acids in this system have a certain faradaic impedance, that will depend on the distance between the ETM and the electrode, their electronic properties, and the composition of the intervening medium, among other things. Of importance in the methods of the invention is that the faradaic impedance between the ETM and the electrode is signficantly different depending on whether the label probes containing the ETMs are specifically or non-specifically bound to the electrode.

Accordingly, the present invention further provides apparatus for the detection of nucleic acids using AC detection methods. The apparatus includes a test chamber which has at least a first measuring or sample electrode, and a second measuring or counter electrode. Three electrode systems are also useful. The first and second measuring electrodes are in contact with a test sample receiving region, such that in the presence of a liquid test sample, the two electrodes may be in electrical contact.

In a preferred embodiment, the first measuring electrode comprises a single stranded nucleic acid capture probe covalently attached via an attachment linker, and a monolayer comprising conductive oligomers, such as are described herein.

The apparatus further comprises an AC voltage source electrically connected to the test chamber; that is, to the measuring electrodes. Preferably, the AC voltage source is capable of delivering DC offset voltage as well.

In a preferred embodiment, the apparatus further comprises a processor capable of comparing the input signal and the output signal. The processor is coupled to the electrodes and configured to receive an output signal, and thus detect the presence of the target nucleic acid.

Thus, the compositions of the present invention may be used in a variety of research, clinical, quality control, or field testing settings.

In a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, or any of the others well known in the art.

In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of

In a preferred embodiment, the arrays are used for mRNA detection. A preferred embodiment utilizes either capture probes or capture extender probes that hybridize close to the 3' polyadenylation tail of the mRNAs. This allows the use of one species of target binding probe for detection, i.e. the probe contains a poly-T portion that will bind to the poly-A tail of the mRNA target. Generally, the probe will contain a second portion, preferably non-poly-T, that will bind to the detection probe (or other probe). This allows one target-binding probe to be made, and thus decreases the amount of different probe synthesis that is done.

In a preferred embodiment, the use of restriction enzymes and ligation methods allows the creation of "universal" arrays. In this embodiment, monolayers comprising capture probes that comprise restriction endonuclease ends, as is generally depicted in Figure 39. By utilizing complementary portions of nucleic acid, while leaving "sticky ends", an array comprising any number of restriction endonuclease sites is made. Treating a target sample with one or more of these restriction endonucleases allows the targets to bind to the array. This can be done without knowing the sequence of the target. The target sequences can be ligated, as desired, using standard methods such as ligases, and the target sequence detected, using either standard labels or the methods of the invention.

The present invention provides methods which can result in sensitive detection of nucleic acids. In a preferred embodiment, less than about 10×10^6 molecules are detected, with less than about 10×10^5 being preferred, less than 10×10^4 being particularly preferred, less than about 10×10^3 being especially preferred, and less than about 10×10^2 being most preferred. As will be appreciated by those in the art, this assumes a 1:1 correlation between target sequences and reporter molecules; if more than one reporter molecule (i.e. electron transfer moeity) is used for each target sequence, the sensitivity will go up.

While the limits of detection are currently being evaluated, based on the published electron transfer rate through DNA, which is roughly 1×10^6 electrons/sec/duplex for an 8 base pair separation (see Meade et al., Angw. Chem. Eng. Ed., 34:352 (1995)) and high driving forces, AC frequencies of about 100 kHz should be possible. As the preliminary results show, electron transfer through these systems is quite efficient, resulting in nearly 100×10^3 electrons/sec, resulting in potential femptoamp sensitivity for very few molecules.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention,

Compound N4. **Compound N3** (6.5 g, 12.8 mmol) was dissolved in 150 mL dry pyridine, followed by adding TMSCl (5.6 g, 51.2 mmol) . The reaction mixture was stirred at room temperature for 1.5 hours. Then phenoxyacetyl chloride (3.3 g, 19.2 mmol) was added at 0 °C. The reaction was then stirred at room temperature for 4 hours and was quenched by the addition of 100 mL water at 0 °C. The solvents were removed under reduced pressure, and the crude gum was further purified by flash chromatography on 90 g of silica gel (1 % methanol/dichloromethane) (2.3 g, 28%).

Compound N5. Compound N4 (2.2 g, 3.4 mmol) and DMAP (200 mg, 1.6 mmol) were dissolved in 150 mL dry pyridine, followed by the addition of DMTCl (1.4 g, 4.1 mmol). The reaction was stirred under argon at room temperature overnight. The solvent was removed under reduced pressure, and the residue was dissolved in 250 mL dichloromethane. The organic solution was washed by 5% $NaHCO_3$ solution (3 x 250 mL) , dried over Na_2SO_4, and concentrated. Further purification by flash chromatography on 55 g of silica gel (1 % TEA/50% hexane/dichloromethane) provided the desired product (1.3 g, 41%).

Compound N6. To a solution of **N5** (3.30 gm, 3.50 mmol) in 150 mL dichloromethane. Diisopropylethylamine (4.87 mL, 8.0 eq.) and catalytic amount of DMAP (200 mg) were added. The mixture was kept at 0 °C, and N, N-diisopropylamino cyanoethyl phosphonamidic chloride (2.34 mL, 10.48 mmol) was added. The reaction mixture was warmed up and stirred at room temperature overnight. After dilution by adding 150 mL of dichloromethane and 250 mL of 5 % $NaHCO_3$ aqueous solution, the organic layer was separated, washed with 5% NaHCO3 (250 mL), dried over Na_2SO_4, and concentrated. The crude product was purified on a flash column of 66 g of silica gel packed with 1% TEA in hexane. The eluting solvents were 1% TEA in hexane (500 mL), 1% TEA and 10% dichloromethane in hexane (500 mL), 1% TEA and 20% dichloromethane in hexane (500 mL). 1% TEA and 50% dichloromethane in hexane (500 mL). Fractions containing the desired products were collected and concentrated to afford the final product (3 gm, 75%).

Example 2

Synthesis of "Branched" nucleoside

The synthesis of **N17** is described as shown in Figure 10.

Synthesis of N14. To a solution of *Tert*-butyldimethylsily chloride (33.38 g, 0.22 mol) in 300 mL of dichloromethane was added imidazole (37.69 g, 0.55 mol) . Immediately, large amount of precipitate was formed. 2-Bromoethanol (27.68 g, 0.22 mol,.) was added slowly at room temperature. The reaction mixture was stirred at this temperature for 3 hours. The organic layer was washed with water

crude product was purified on a flash column of 120 gm of silica gel packed with 1% TEA and 10% dichloromethane in hexane. The eluting solvents were 1% TEA and 10% dichloromethane in hexane (500 mL), 1% TEA and 20% dichloromethane in hexane (500 mL), and 1% TEA and 40% dichloromethane in hexane (1500 mL). The right fractions were collected and concentrated to afford the final product (7.37gm, 60%).

Example 3

Synthesis of nucleoside with ferrocene attached via a phosphate

The synthesis of **Y63** is described as shown in Figure 11.

Synthesis of C102: A reaction mixture consisting of 10.5gm (32.7 mmol) of **N2**, 16gm of potassium acetate and 350 ml of DMF was stirred at 100°C for 2.5hrs. The reaction mixture was allowed to cool to room temperature and then poured into a mixture of 400ml of ether and 800ml of water. The mixture was shaken and the organic layer was separated. The aqueous layer was extracted twice with ether. The combined ether extracts were dried over sodium sulfate and then concentrated for column chromatography. Silica gel(160 gm) was packed with 1% TEA/Hexane. The crude was loaded and the column was eluted with 1 % TEA/0-100 % CH_2Cl_2/Hexane. Fractions containing desired product were collected and concentrated to afford 5.8g (59.1 %) of **C102**.

Synthesis of Y61: To a flask containing 5.1gm (17.0 mmol) of **C102** was added 30ml of Dioxane. To this solution, small aliquots of 1M NaOH was added over a period of 2.5 hours or until hydrolysis was complete. After hydrolysis the product was extracted using hexane. The combined extracts were dried over sodium sulfate and concentrated for chromatography. Silica gel (100 gm) was packed in 10% EtOAc/ Hexane. The crude product solution was loaded and the column was eluted with 10% to 50% EtOAc in hexane. The fractions containing desired product were pooled and concentrated to afford 4.20 gm (96.1 %) of **Y61**.

Synthesis of Y62: To a flask containing 4.10 gm (15.9 mmol) of **Y61** was added 200ml of dichloromethane and 7.72 ml of DIPEA and 4.24 gm (15.9 mmol) of bis(diisopropylamino) chlorophosphine. This reaction mixture was stirred under the presence of argon overnight. After the reaction mixture was concentrated to 1/3 of its original volume, 200ml of hexane was added and then the reaction mixture was again concentrated to 1/3 is original volume. This procedure was repeated once more. The precipitated salts were filtered off and the solution was concentrated to afford 8.24gm of crude **Y62**. Without further purification, the product was used for next step.

Synthesis of W69: A solution of 5.2 gm (23.6 mmol)of 4-iodophenol in 50 ml of dry DMF was cooled in an ice-water bath under Ar. To the mixture was added 1.0 gm of NaH (60% in mineral oil, 25.0 mmol) portion by portion. The mixture was stirred at the same temperature for about 35 min. and at room temperature for 30 min. A solution of 8.68 gm (19.2 mmol) of **W68** in 20 ml of DMF was added to the flask under argon. The mixture was stirred at 50 °C for 12 hr with the flask covered with aluminum foil. DMF was removed under reduced pressure. The residue was dissolved in 300 ml of ethyl acetate, and the solution was washed with H_2O (6 x 50 mL). Ethyl acetate was removed under reduced pressure and the residue was loaded into a 100 g silica gel column packed with 30 % CH_2Cl_2/hexane for the purification. The column was eluted with 30-100% CH_2Cl_2/hexane. The fractions containing the desired product were pooled and concentrated to afford 9.5 gm (84.0 %) of the title product.

Synthesis of W70: To a 100 ml round bottom flask containing 6.89 gm (11.6 mmol) of **W69** was added 30 ml of 1M TBAF THF solution. The solution was stirred at room temperature for 5h. THF was removed and the residue was dissolved 150 ml of CH_2Cl_2. The solution was washed with H_2O (4 x 25 mL). Removal of solvent gave 10.5 gm of semi-solid. Silica gel (65 gm) was packed with 50 % CH_2Cl_2/hexane, upon loading the sample solution, the column was eluted with 0-3 % CH_3OH/CH_2Cl_2. The fractions were identified by TLC (CH_3OH : CH_2Cl_2 = 5 : 95). The fractions containing the desired product were collected and concentrated to afford 4.10 gm (99.0%) of the title product.

Synthesis of W71: To a flask was added 1.12 gm (3.18 mmol) of **W70**, 0.23 g (0.88 mmol) of PPh_3, 110 mg (0.19 mmol) of $Pd(dba)_2$, 110 mg (0.57 mmol) of CuI and 0.75g (3.2 mmol) of **Y4** (one unit wire). The flask was flushed with argon and then 65 ml of dry DMF was introduced, followed by 25 ml of diisopropylamine. The mixture was stirred at 55 °C for 2.5 h. All tsolvents were removed under reduced pressure. The residue was dissolved in 100 ml of CH_2Cl_2, and the solution was thoroughly washed with the saturated EDTA solution (2 x 100 mL). The Removal of CH_2Cl_2 gave 2.3 g of crude product. Silica gel (30 gm) was packed with 50 % CH_2Cl_2/hexane, upon loading the sample solution, the column was eluted with 10 % ethyl acetate/CH_2Cl_2. The concentration of the fractions containing the desired product gave1.35 gm (2.94 mmol) of the title product, which was further purified by recrystallization from hot hexane solution as colorless crystals.

Example 5

Synthesis of nucleoside attached to an insulator

As shown in Figure 13.

Synthesis of C108: To a flask was added 2.0gm (3.67 mmol) of 2'-amino-5'-O-DMT uridine, 1.63gm (3.81 mmol) of **C44**, 5ml of TEA and 100ml of dichloromethane. This reaction mixture was stirred at

allowed to cool to RT and 50 ml of degassed 1.0 M NaOH aqueous solution were injected. Additional refluxing for 3 hrs required for reaction completion. Resulted reaction mixture was cooled with ice bath and poured, with stirring, into a vessel containing 200 ml of ice water. This mixture was titrated to pH=7 by 1.0 M HCl and extracted with 300 ml of ether. The organic layer was separated, washed with 3x150 ml of water, 150 ml of saturated NaCl aqueous solution and dried over sodium sulfate. After removal of ether material was purified by recrystalization from n-hexane, filtering out and drying over high vacuum. 5.1 gr (97% yield) of the desired product was obtained.

16-Bromohexadecan-1-ol. Under inert atmosphere 10 ml of BH_3·THF complex (1.0 M THF solution) were added to 30 ml THF solution of 2.15 gr (6.41 mmole) of 16-bromohexadecanoic acid at -20°C. Reaction mixture was stirred at this temperature for 2 hrs and then additional 1 hr at RT. After that time the resulted mixture was poured, with stirring, into a vessel containing 200 ml of ice/saturated sodium bicarbonate aqueous solution. Organic compounds were extracted with 3x200 ml of ether. The ether fractions were combined and dried over sodium sulfate. After removal of ether material was dissolved in minimum amount of dicloromethane and purified by silica gel chromatography (100% dicloromethane as eluent). 1.92 gr (93% yield) of the desired product were obtained.

16-Mercaptohexadecan-1-ol. Under inert atmosphere 365 mg of sodium metal suspension (40% in mineral oil) were added dropwise to 20 ml of dry methanol at 0°C. After completion of addition the reaction mixture was stirred for 10 min at RT followed by addition of 0.45 ml (6.30 mmole) of thioacetic acid. After additional 10 min of stirring 3 ml degassed methanolic solution of 1.0 gr (3.11 mmole) of 16-bromohexadecan-1-ol were added. The resulted mixture was refluxed for 15 hrs, allowed to cool to RT and 20 ml of degassed 1.0 M NaOH aqueous solution were injected. The reaction completion required additional 3 hr of reflux. Resulted reaction mixture was cooled with ice bath and poured, with stirring, into a vessel containing 200 ml of ice water. This mixture was titrated to pH=7 by 1.0 M HCl and extracted with 300 ml of ether. The organic layer was separated, washed with 3x150 ml of water, 150 ml of saturated NaCl aqueous solution and dried over sodium sulfate. After ether removal material was dissolved in minimum amount of dicloromethane and purified by silica gel chromatography (100% dicloromethane as eluent). 600 mg (70% yield) of the desired product were obtained.

A clean gold covered microscope slide was incubated in a solution containing 100 micromolar HS-$(CH_2)_{16}$-COOH in ethanol at room temperature for 4 hours. The electrode was then rinsed throughly with ethanol and dried. 20-30 microliters of wire-1 + wire-2 solution (1 micromolar in 1XSSC buffer at pH 7.5) was applied to the electrode in a round droplet. The electrode was incubated at room temperature for 4 hours in a moist chamber to minimize evaporation. The wire-1 solution was then

A variety of different ETM attachments as depicted in Figure 15 were compared. As shown in Table 1, a detection probe was attached to the electrode surface (the sequence containing the wire in the table). Positive (i.e. probes complementary to the detection probe) and negative (i.e. probes not complementary to the detection probe) control label probes were added.

Electrodes containing the different compositions of the invention were made and used in AC detection methods. The experiments were run as follows. A DC offset voltage between the working (sample) electrode and the reference electrode was swept through the electrochemical potential of the ferrocene, typically from 0 to 500 mV. On top of the DC offset, an AC signal of variable amplitude and frequency was applied. The AC current at the excitation frequency was plotted versus the DC offset.

The results are shown in Table 2, with the Y63, VI and IV compounds showing the best results.

Metal Complexes	Redox Potential (mV)	10 Hz	100 Hz	1,000 Hz	10,000 Hz
I	400	Not clear	Not clear	Not clear	Not clear
II	350	0.15 μA	0.01 μA	0.005 μA	ND
III (+ control)	360	0.025 μA	0.085 μA	0.034 μA	ND
III (- control)	360	0.022 μA	0.080 μA	0.090 μA	ND
IV	140	0.34 μA	3.0 μA	13.0 μA	35 μA
V	400	0.02 μA	ND	0.15 μA	ND
VI(1)	140	0.22 μA	1.4 μA	4.4 μA	8.8 μA
VI(2)	140	0.22 μA	0.78 μA	5.1 μA	44 μA
VII	320	0.04 μA	ND	0.45 μA	No Peak
VIII(not purified)	360	0.047 μA	ND	ND	No Peak
Y63	160	.25 μA	ND	36 μA	130 μA

Not clear: There is no difference between positive control and negative control.
ND: Not determined

Table of the Oligonucleotides Containing Different Metal Complexes

Metal Complexes	**Positive Control Sequence Containing Metal Complexes and Numbering**	**Negative Control Sequence Containing Metal Complexes and Numbering**
I	5'-A(I)C (I)GA GTC CAT GGT-3' #D199_1	5'-A(I)G (I)CC TAG CTG GTG-3' #D200_1

A variety of systems have been run and shown to work well, as outlined below. All compounds are referenced in the Figures. Generally, the systems were run as follows. The surfaces were made, comprising the electrode, the capture probe attached via an attachment linker, the conductive oligomers, and the insulators, as outlined above. The other components of the system, including the target sequences, the capture extender probes, and the label probes, were mixed and generally annealed at 90°C for 5 minutes, and allowed to cool to room temperature for an hour. The mixtures were then added to the electrodes, and AC detection was done.

Use of a capture probe, a capture extender probe, an unlabeled target sequence and a label probe:

A capture probe **D112**, comprising a 25 base sequence, was mixed with the **Y5** conductive oligomer and the **M44** insulator at a ratio of 2:2:1 using the methods above. A capture extender probe **D179**, comprising a 24 base sequence perfectly complementary to the **D112** capture probe, and a 24 base sequence perfectly complementary to the **2tar** target, separated by a single base, was added, with the **2tar** target. The **D179** molecule carries a ferrocene (using a C15 linkage to the base) at the end that is closest to the electrode. When the attachment linkers are conductive oligomers, the use of an ETM at or near this position allows verification that the **D179** molecule is present. A ferrocene at this position has a different redox potential than the ETMs used for detection. A label probe **D309** (dendrimer) was added, comprising a 18 base sequence perfectly complementary to a portion of the target sequence, a 13 base sequence linker and four ferrocenes attached using a branching configuration. A representative scan is shown in Figure 16A. When the **2tar** target was not added, a representative scan is shown in Figure 16B. No further representative scans are shown.

Use of a capture probe and a labeled target sequence:

Example A: A capture probe **D94** was added with the **Y5** and **M44** conductive oligomer at a 2:2:1 ratio with the total thiol concentration being 833 µM on the electrode surface, as outlined above. A target sequence (**D336**) comprising a 15 base sequence perfectly complementary to the **D94** capture probe, a 14 base linker sequence, and 6 ferrocenes linked via the **N6** compound was used. A representative scan is shown in Figure 20C. The use of a different capture probe, **D109**, that does not have homology with the target sequence, served as the negative control.

Example B: A capture probe **D94** was added with the **Y5** and **M44** conductive oligomer at a 2:2:1 ratio with the total thiol concentration being 833 µM on the electrode surface, as outlined above. A target sequence (**D429**) comprising a 15 base sequence perfectly complementary to the **D94** capture probe, a **C131** ethylene glycol linker hooked to 6 ferrocenes linked via the **N6** compound was used. A representative scan is shown in Figure 20E. The use of a different capture probe, **D109**, that does not have homology with the target sequence, served as the negative control.

complementary to the capture probe resulted in no signal; similarly, the removal of **MT1** resulted in no signal.

Example B: A capture probe **D334**, **Y5** conductive oligomer and the **M44** insulator were put on the electrode at 2:2:1 ratio with the total thiol concentration being 833 μM. A target sequence **LP280** was added, that comprises a sequence complementary to the capture probe and a 20 base sequence complementary to the label probe **D335** were combined; in this case, the label probe **D335** was added to the target prior to introduction to the electrode. The label probe contains six ferrocenes attached using the **N6** linkages depicted in the Figures. Replacing **LP280** with the **LN280** probe (which is complementary to the label probe but not the capture probe) resulted in no signal.

12. A composition according to claim 1 wherein said capture binding ligand is a carbohydrate.

13. A composition according to claim 2, 6 or 7 wherein said recruitment linker is nucleic acid.

14. A method of detecting a target analyte in a sample comprising:

a) binding a target analyte to an electrode comprising:

i) a monolayer comprising a mixture of conductive oligomers and insulators; and

ii) a covalently attached capture binding ligand;

b) binding a solution binding ligand to said target analyte, wherein said solution binding ligand comprises a first portion that will bind to a target analyte and a directly or indirectly attached recruitment linker comprising a first portion comprising at least one ETM; and

d) detecting the presence of said ETM using said electrode as an indication of the presence of the target analyte.

15. A method of detecting a target analyte in a sample comprising:

a) replacing said target analyte in said sample with a target analyte analog comprising a directly or indirectly attached recruitment linker comprising a first portion comprising at least one ETM;

b) binding said target analyte analog to an electrode comprising:

i) a monolayer comprising a mixture of conductive oligomers and insulators; and

ii) a covalently attached capture binding ligand;

d) detecting the presence of said ETM using said electrode as an indication of the presence of the target analyte.

16. A method according to claim 14 or 15 wherein said recruitment linker comprises a plurality of ETMs.

17. A method according to claim 14 or 15 wherein said ETM is ferrocene.

18. A method according to claim 14 or 15 wherein said capture binding ligand is nucleic acid.

19. A method according to claim 14 or 15 wherein said capture binding ligand is a protein.

20. A method according to claim 14 or 15 wherein said capture binding ligand is a carbohydrate.

21. A method according to claim 14 or 15 wherein said recruitment linker is nucleic acid.

FIG._2A

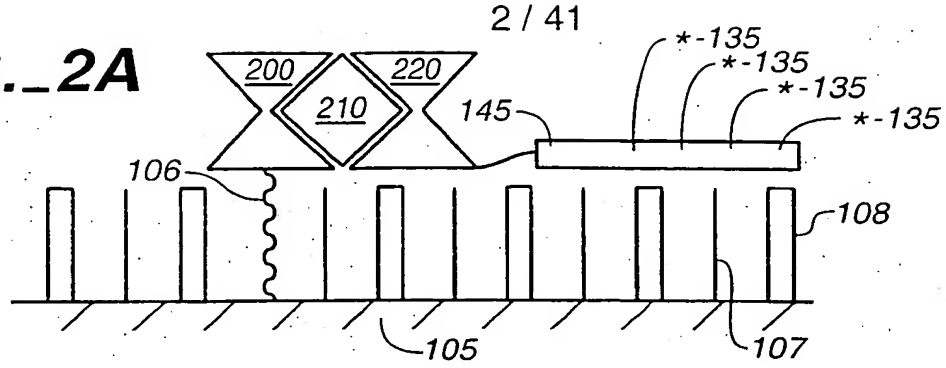


FIG._2B

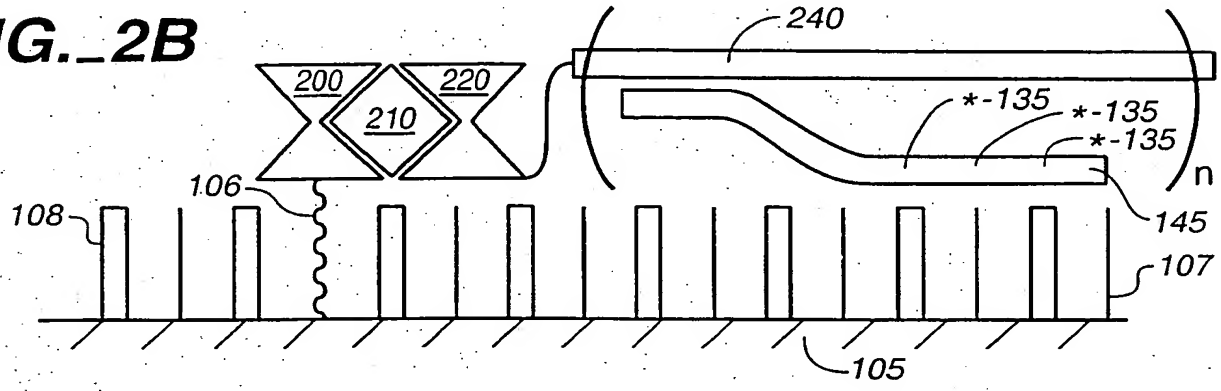


FIG._2C

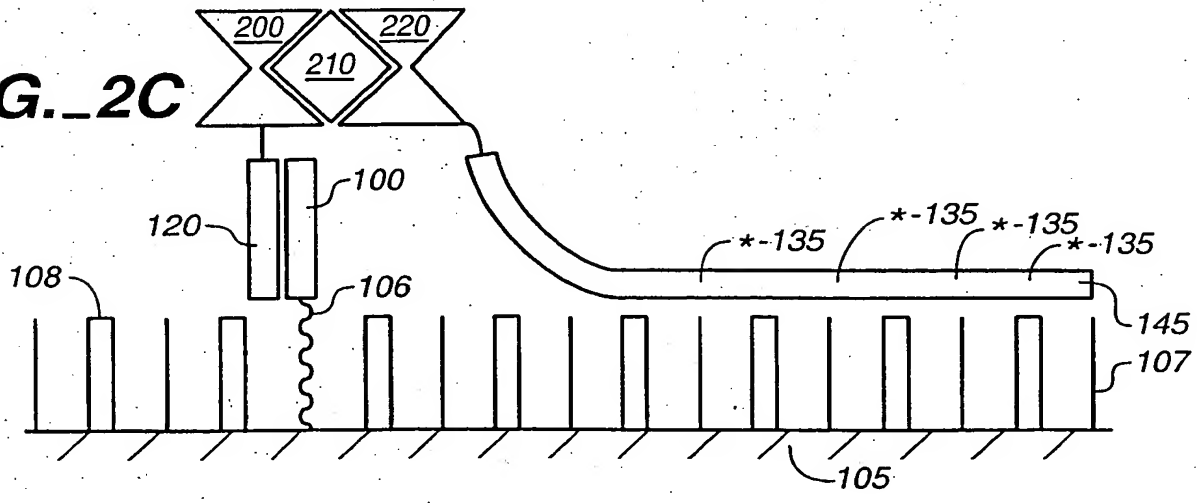
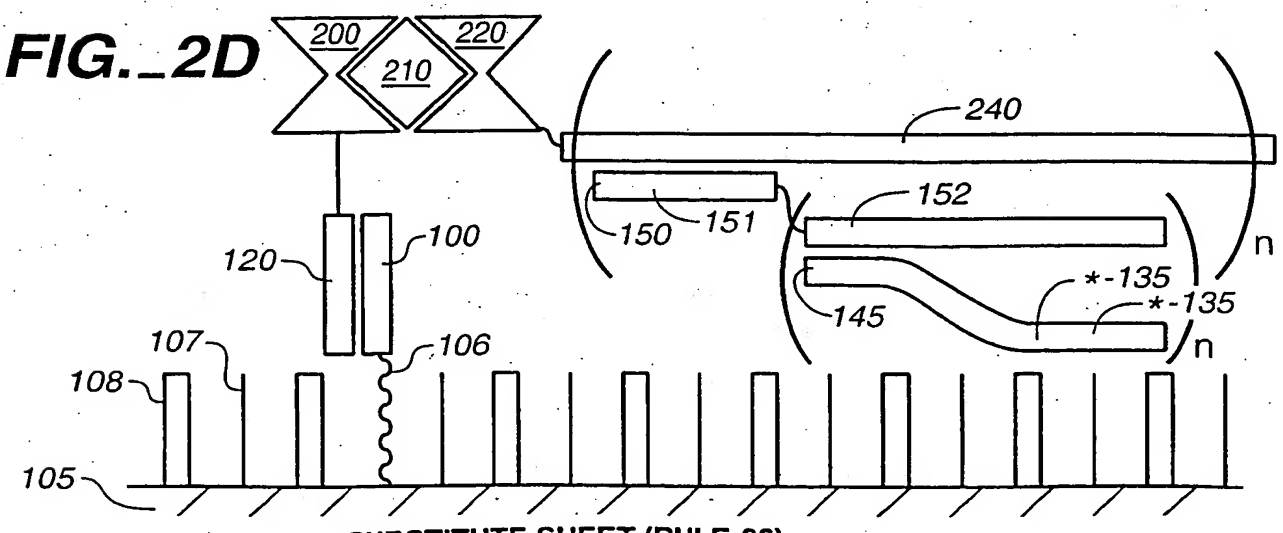


FIG._2D



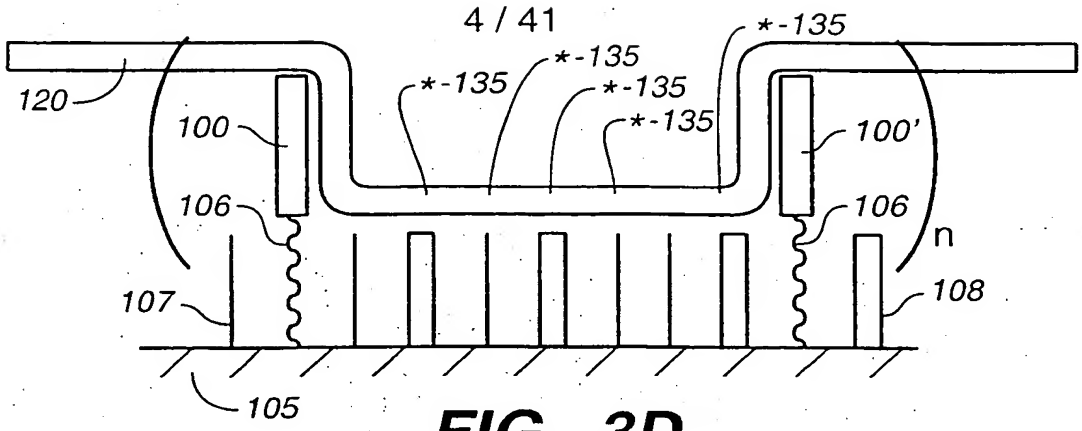


FIG._3D

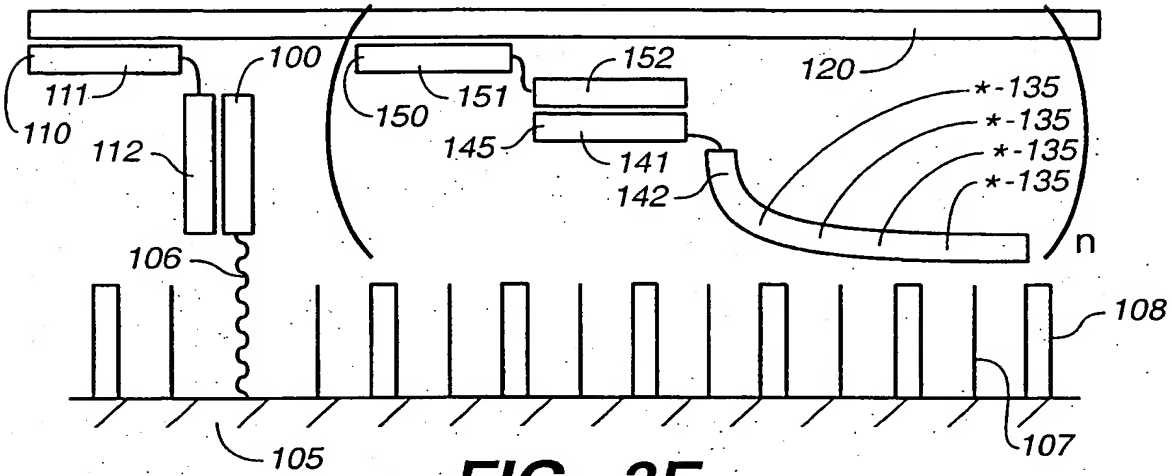


FIG._3E

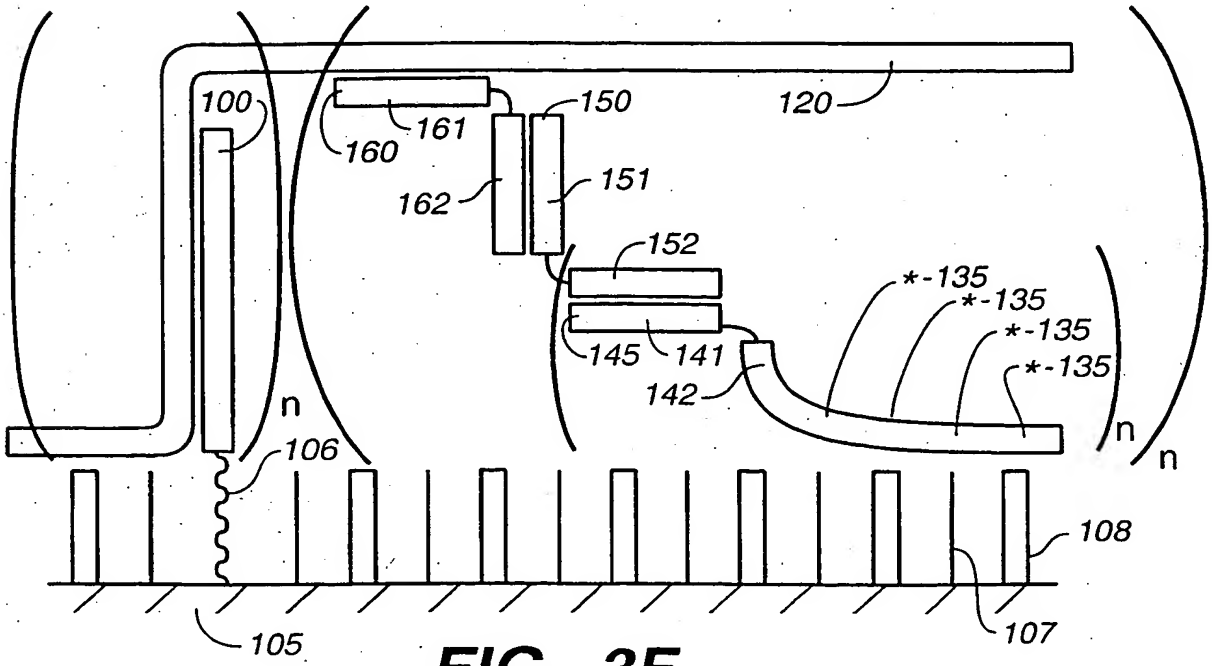


FIG._3F

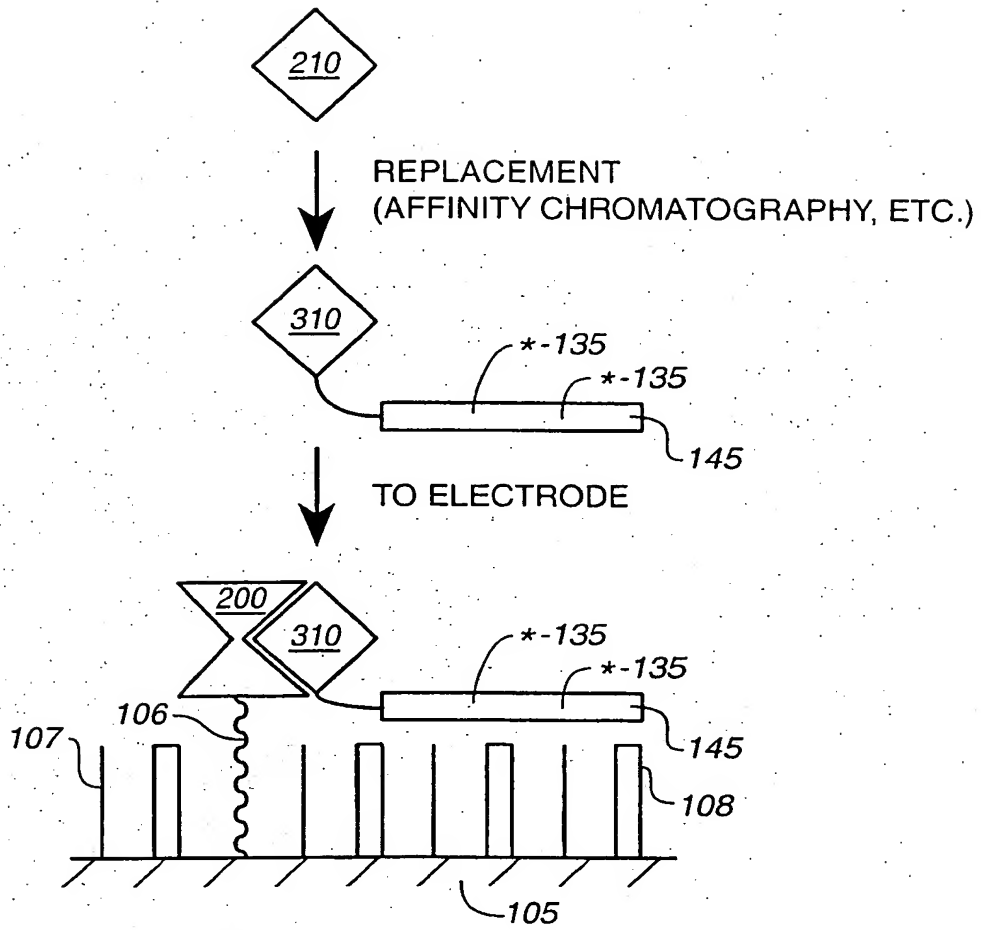


FIG._4A

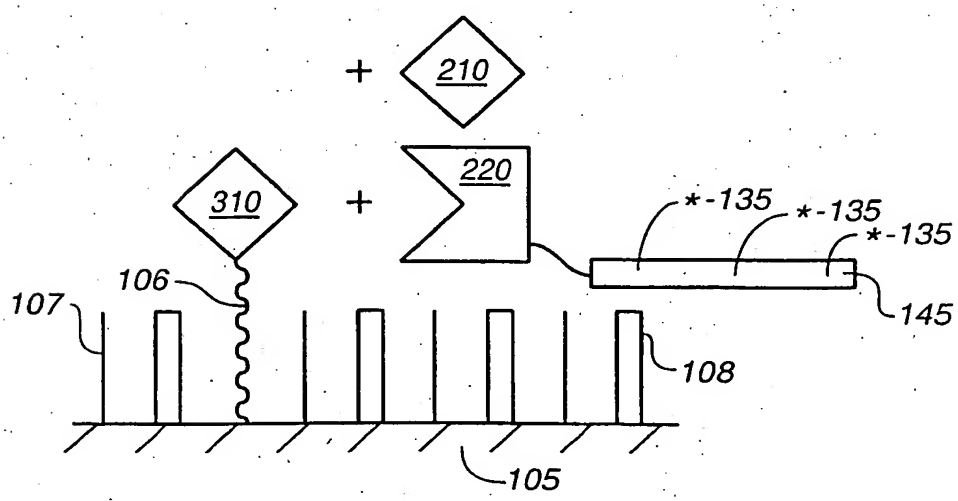


FIG._4B

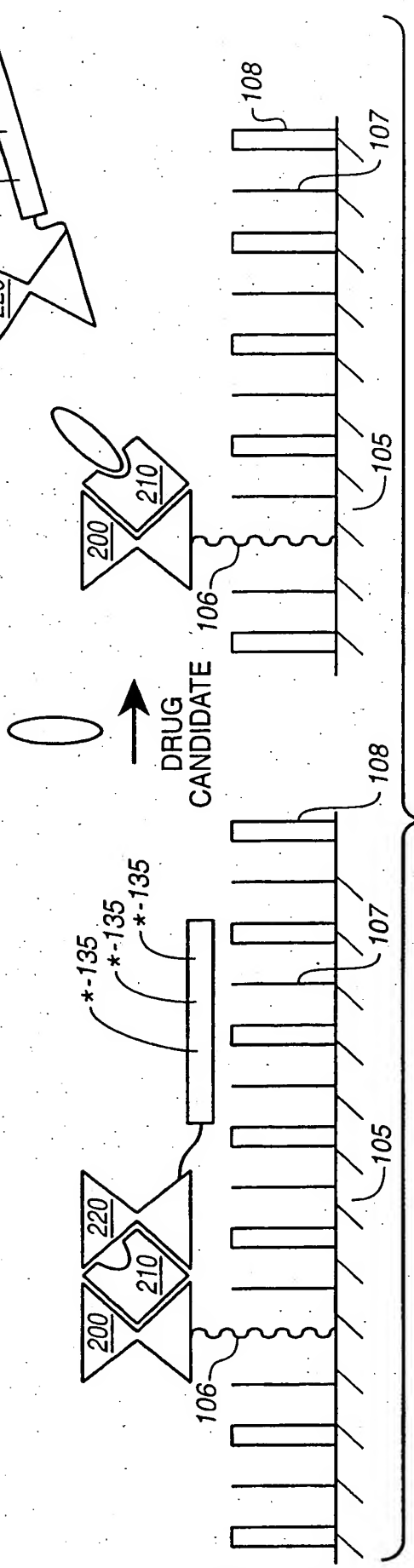


FIG._5B

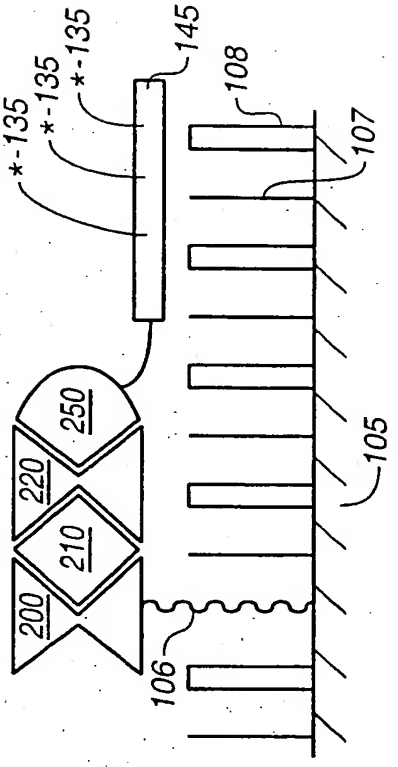


FIG._5E

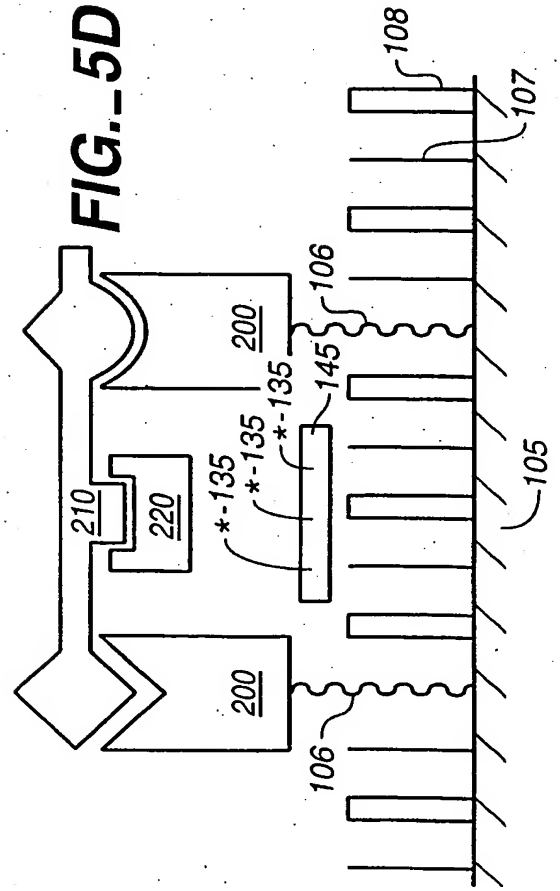


FIG._5D

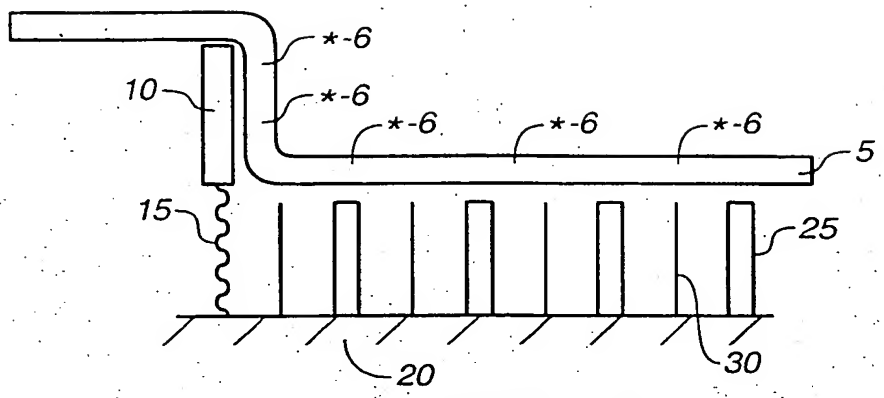


FIG._6A

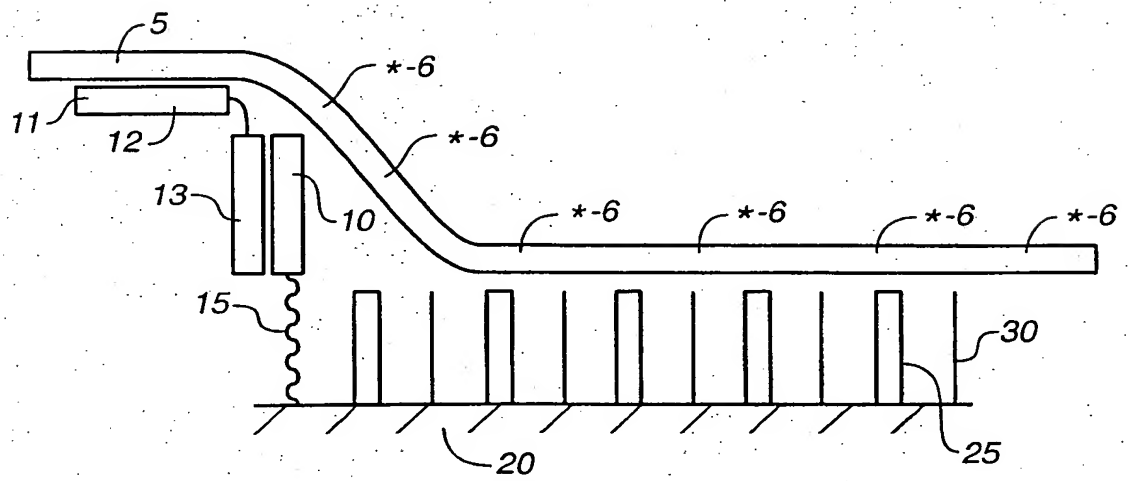


FIG._6B

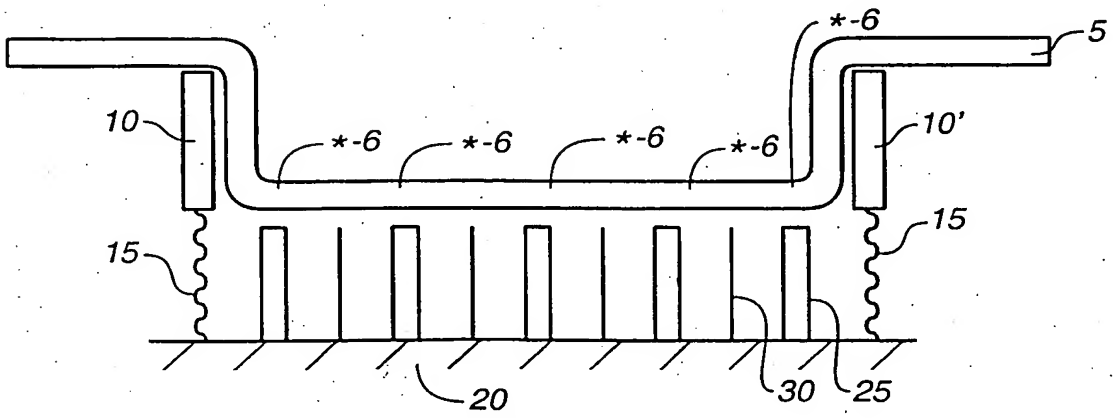


FIG._6C

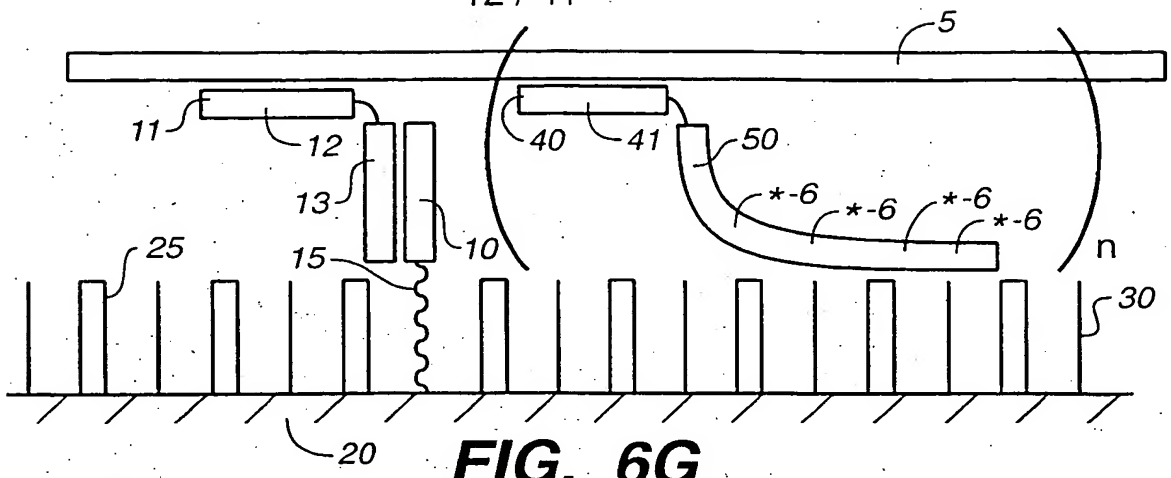


FIG._6G

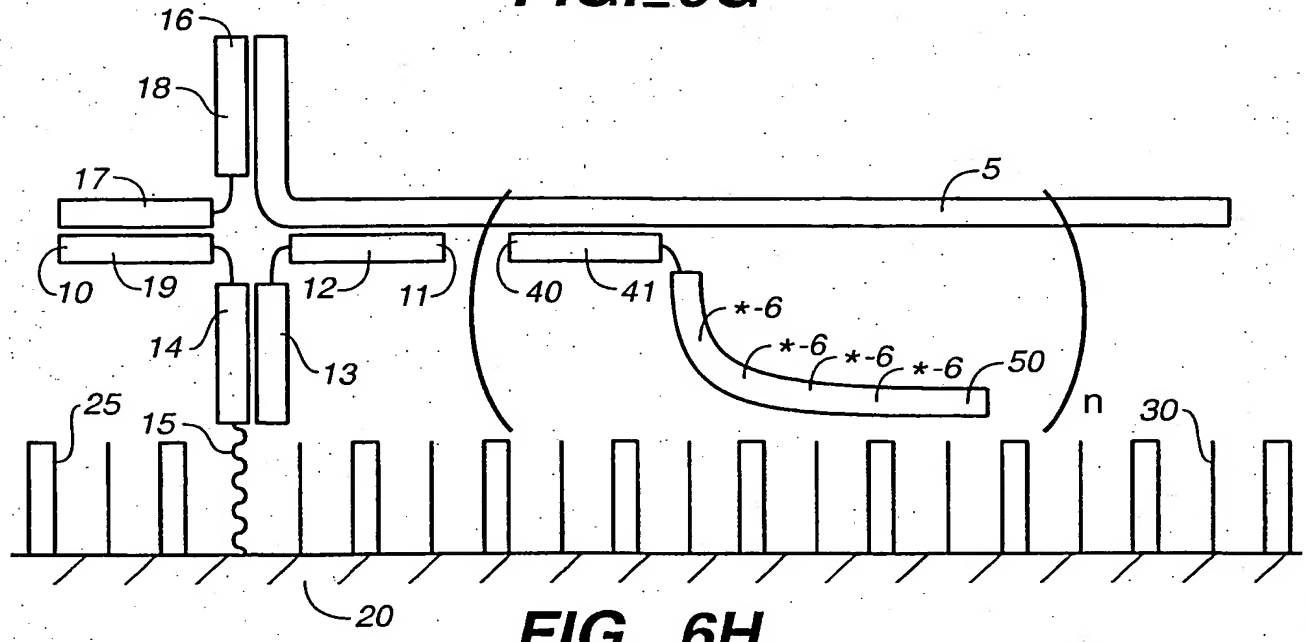


FIG._6H

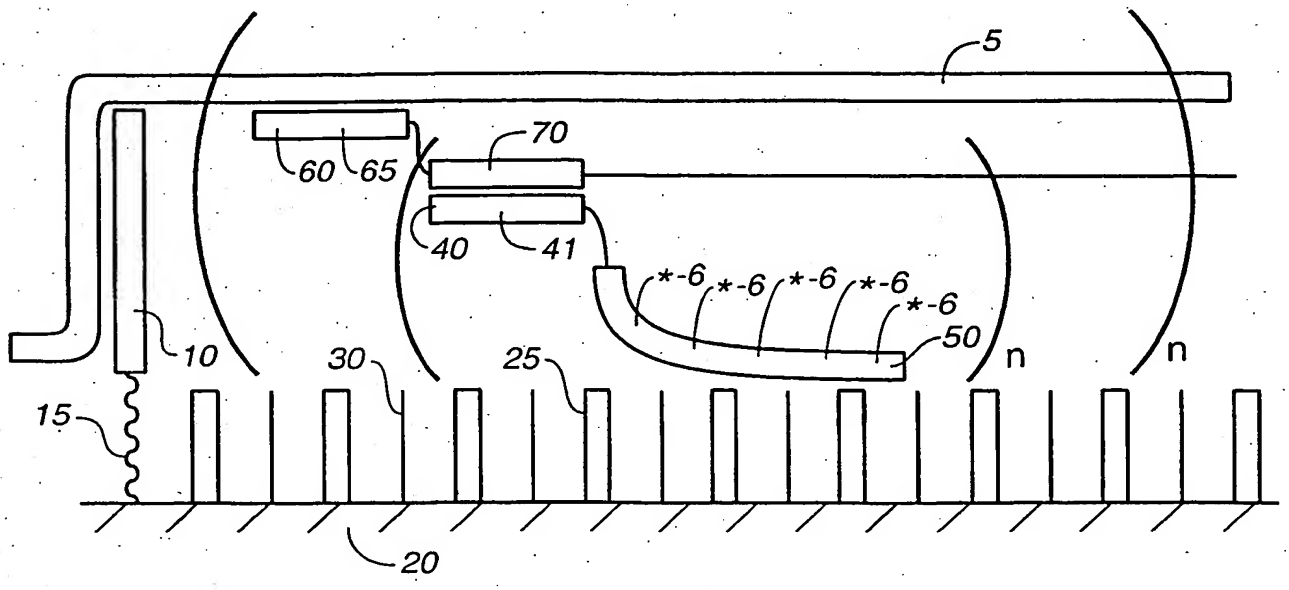


FIG._6I

FIG._6L

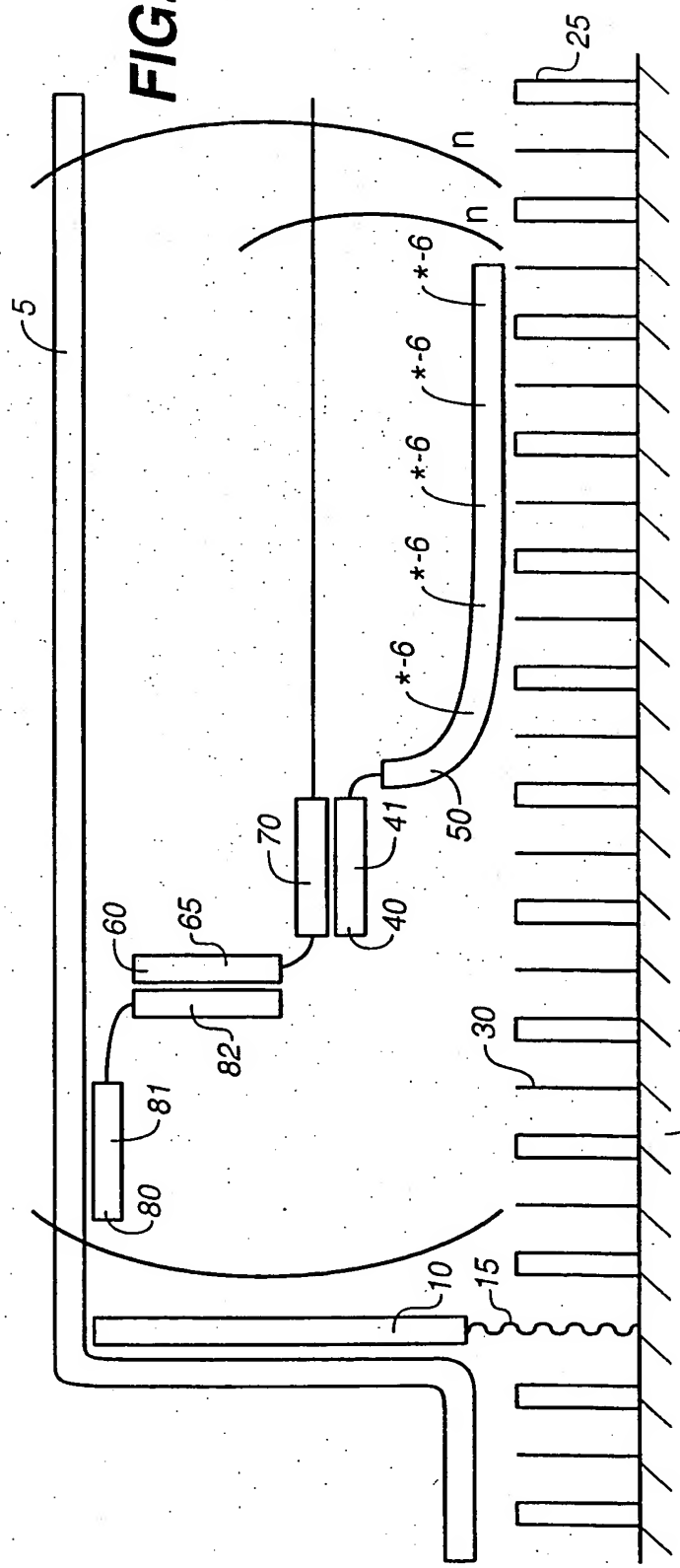


FIG._6P

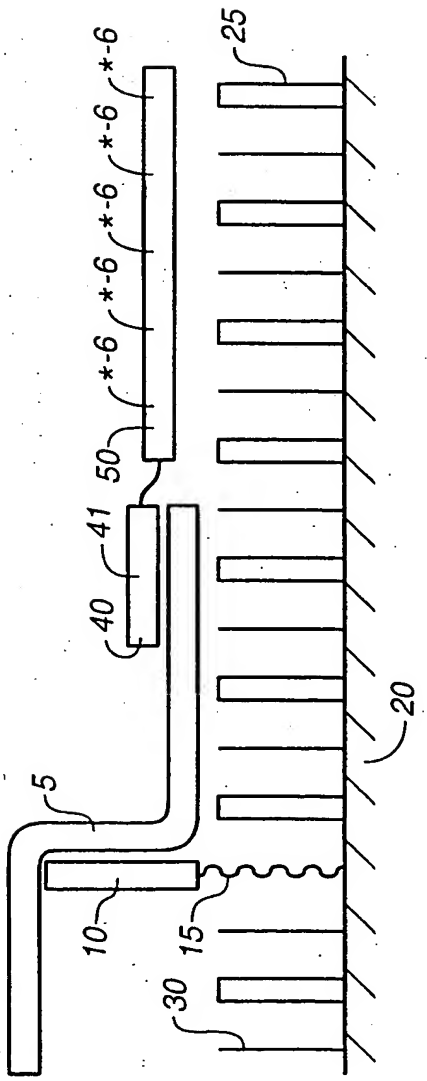


FIG._6N

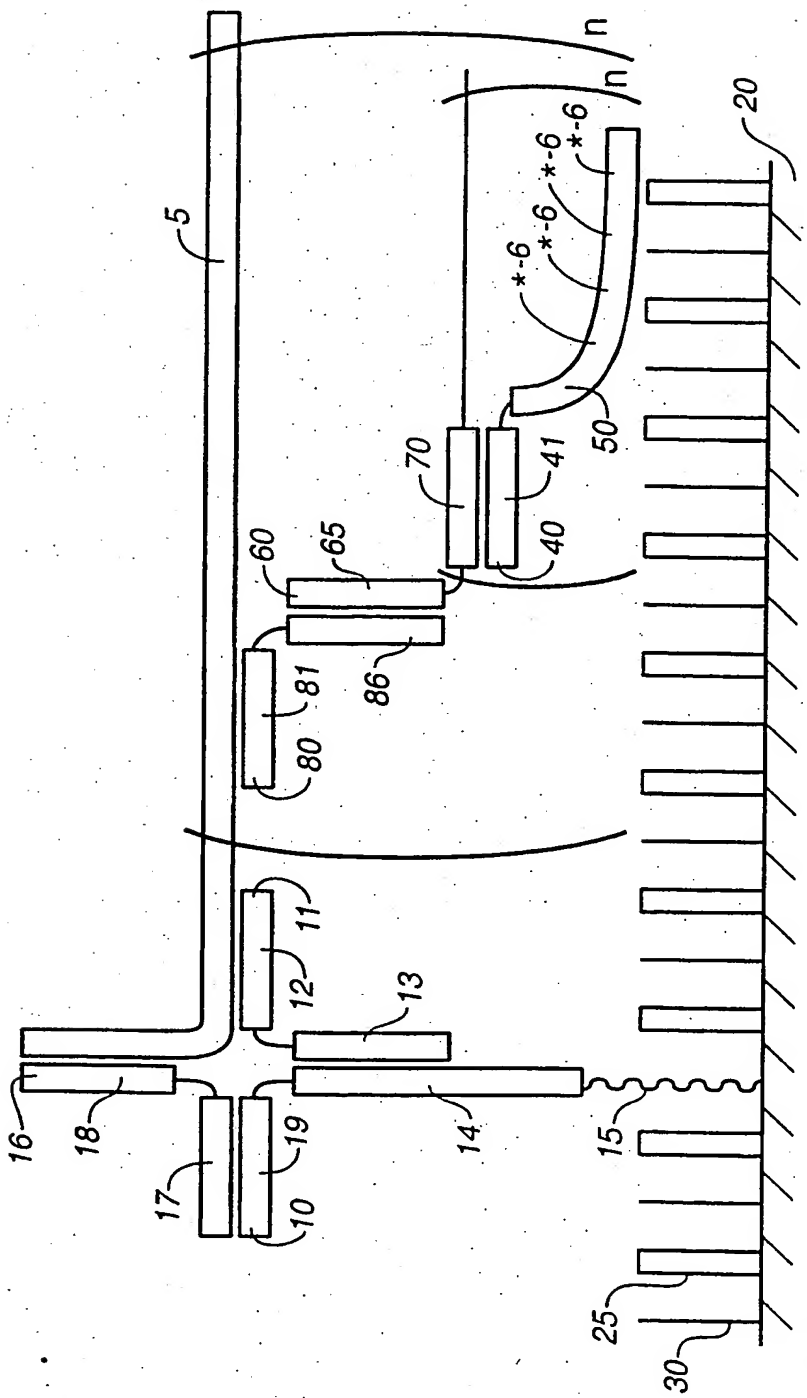
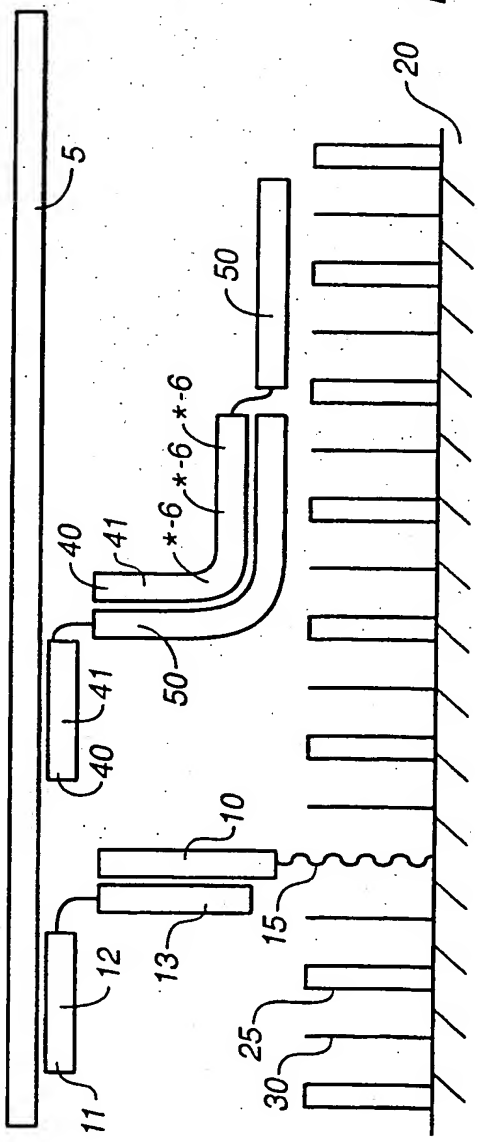


FIG._6R



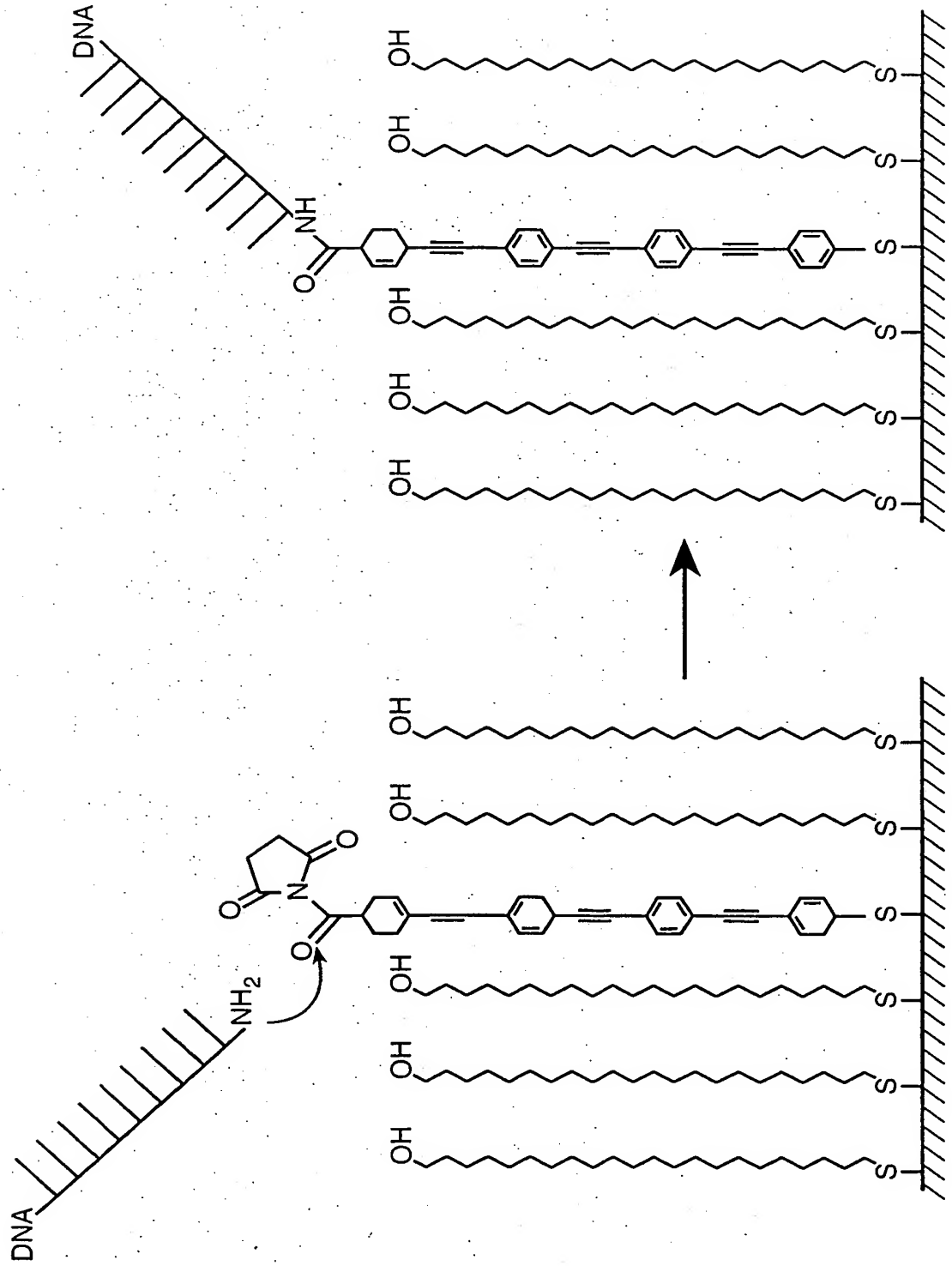


FIG._7

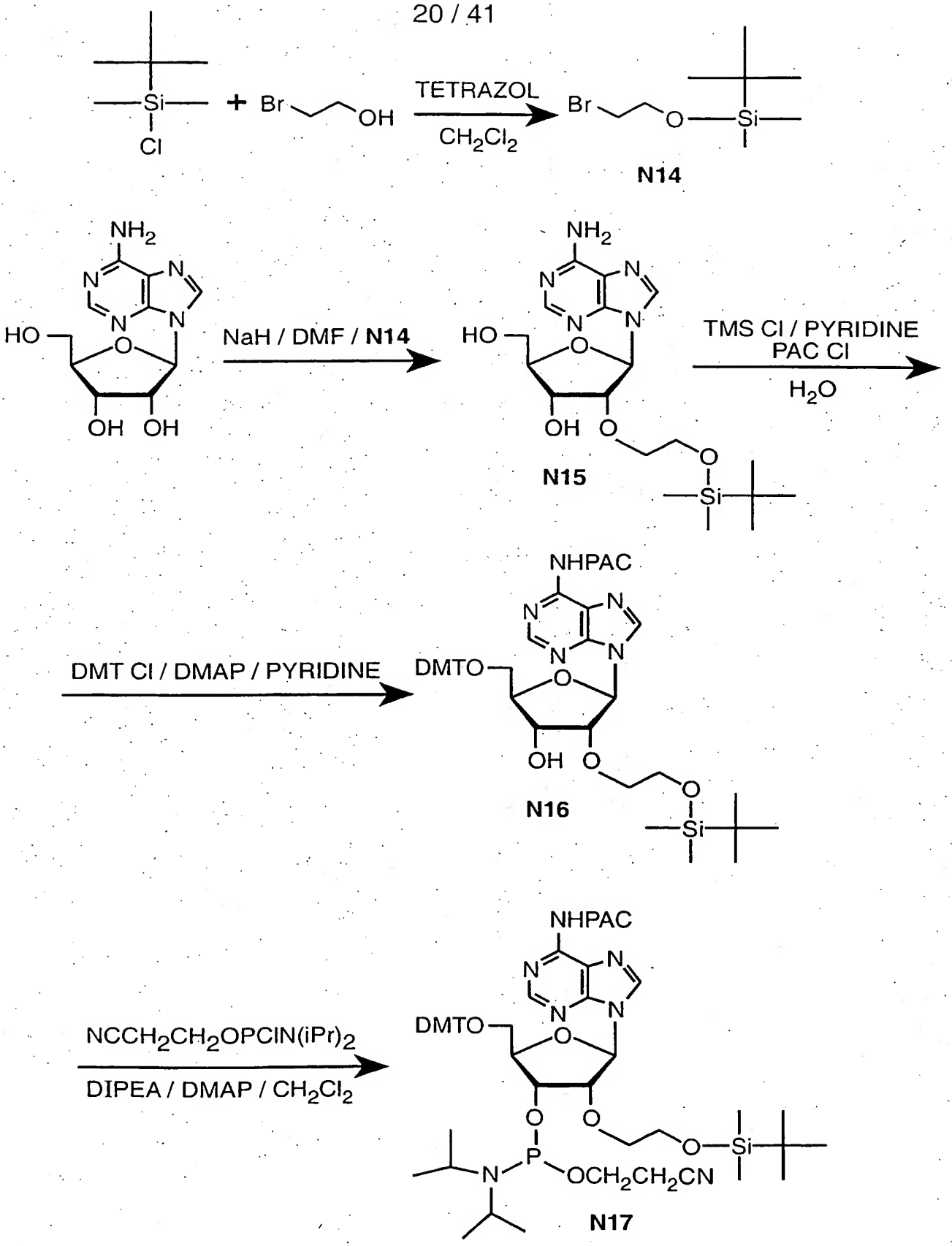


FIG._10

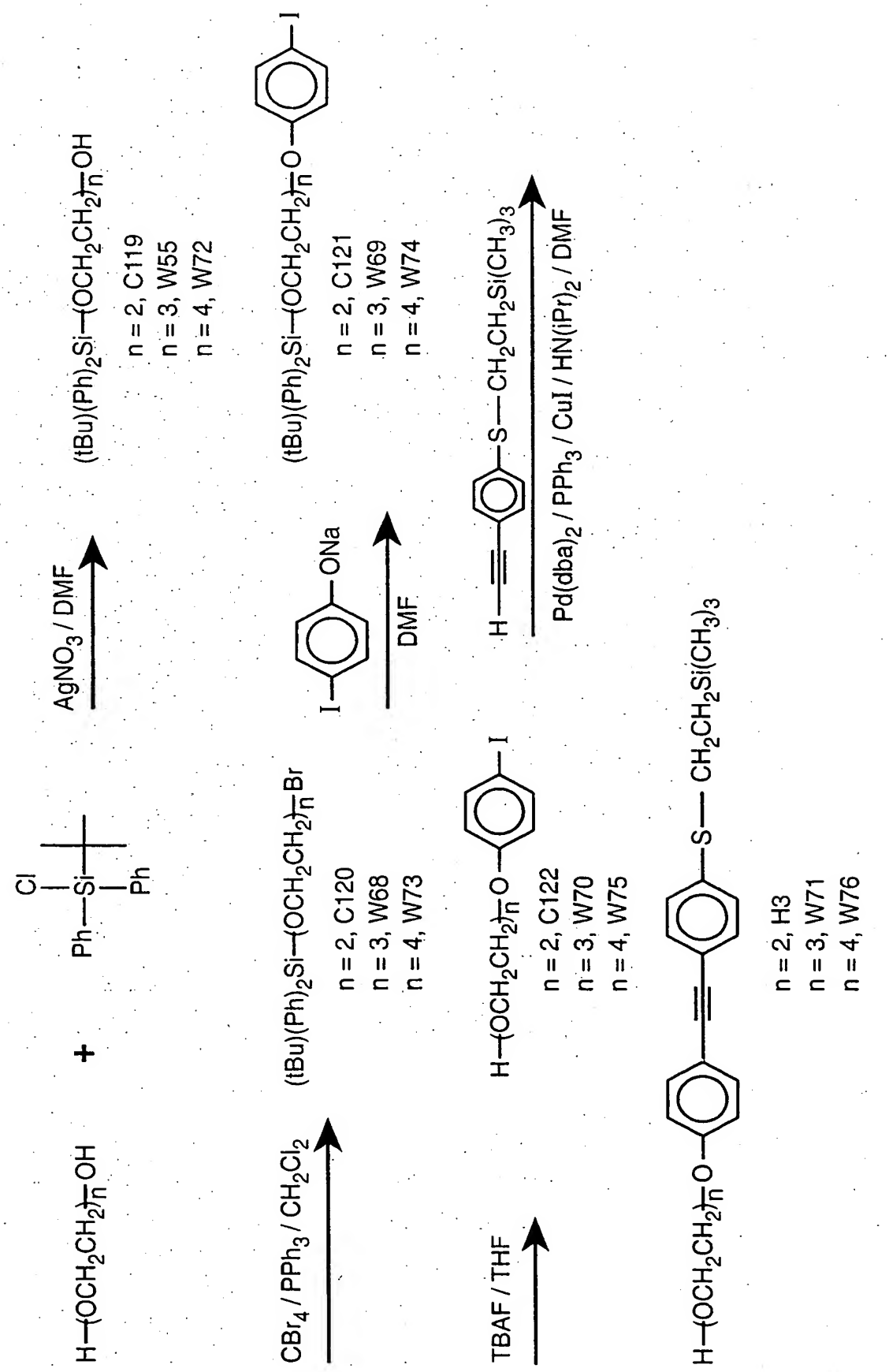


FIG._12

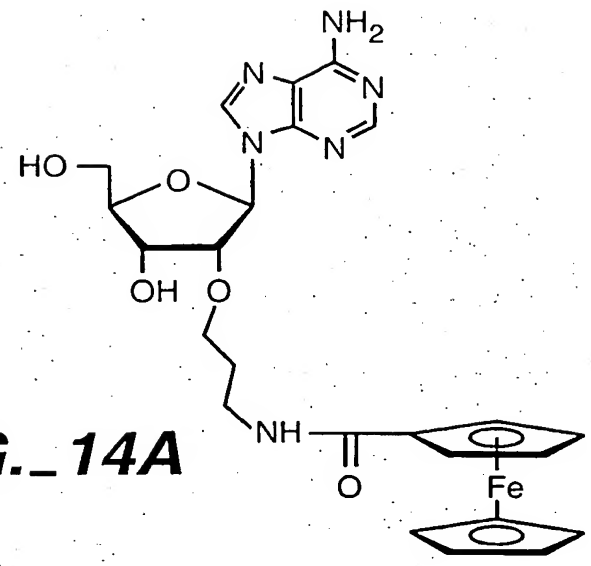


FIG._14A

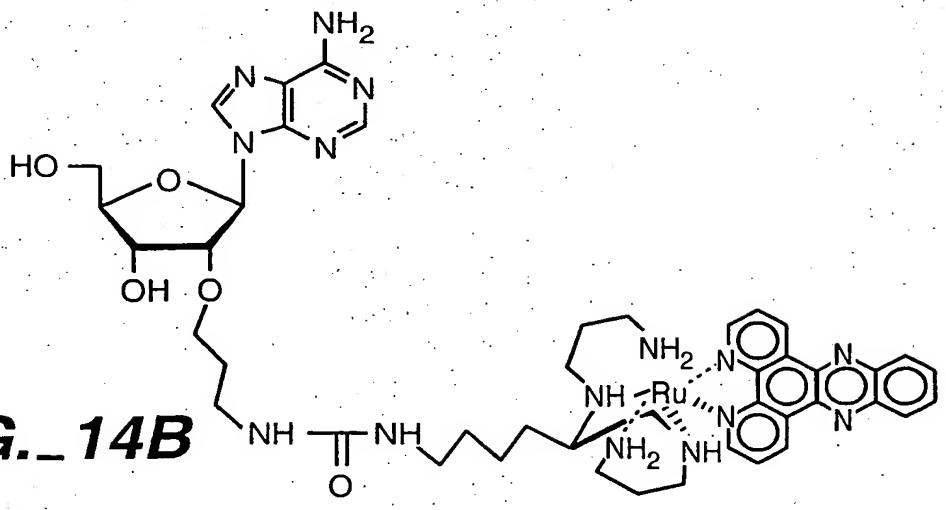


FIG._14B

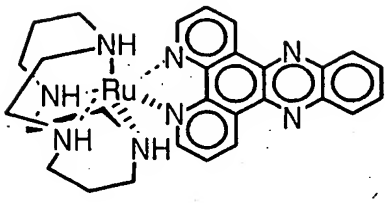


FIG._14C

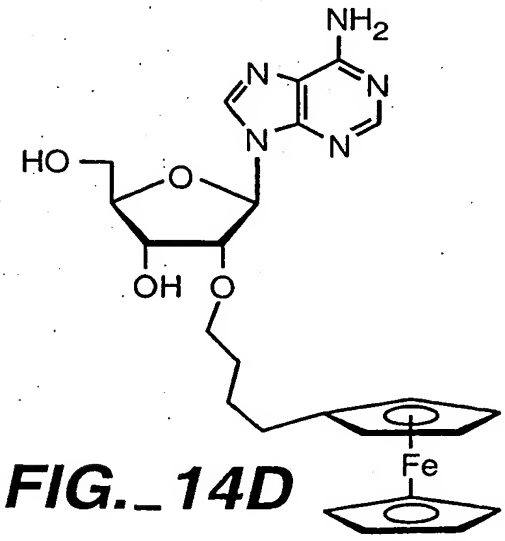


FIG._14D

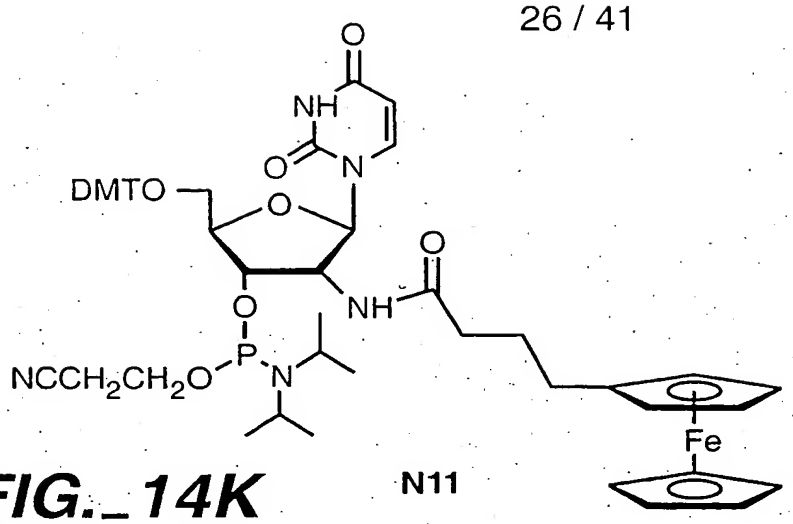


FIG._14K

N11

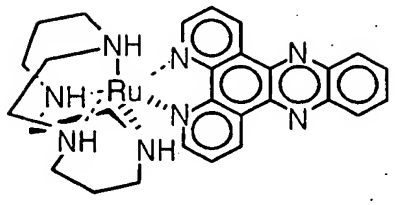


FIG._15C

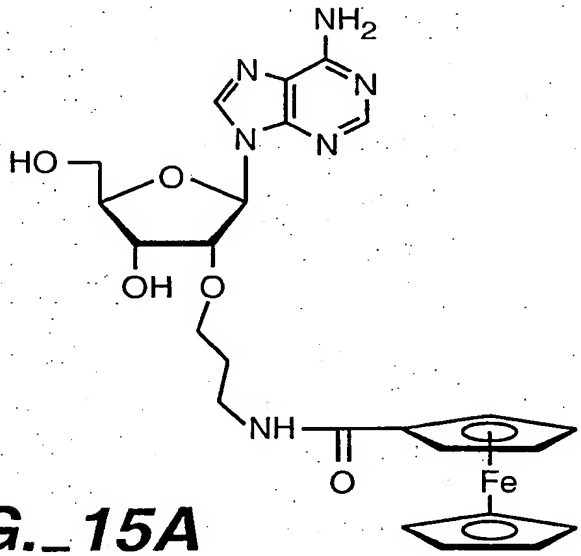
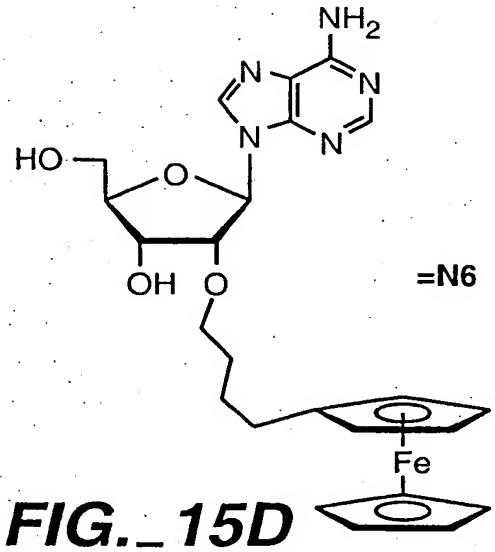


FIG._15A



=N6

FIG._15D

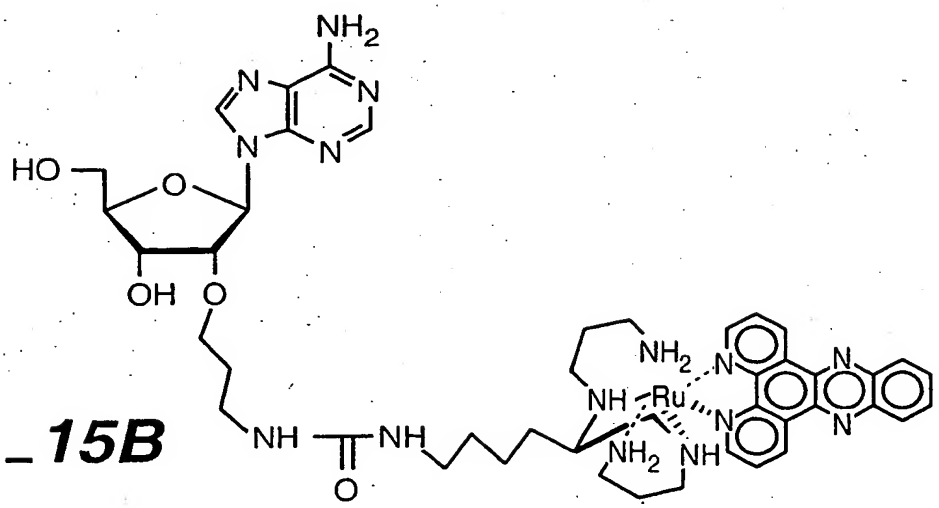


FIG._15B

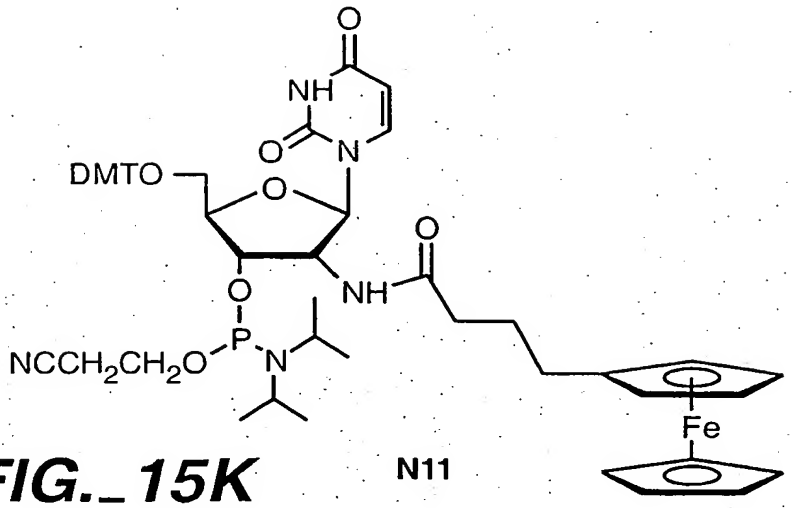
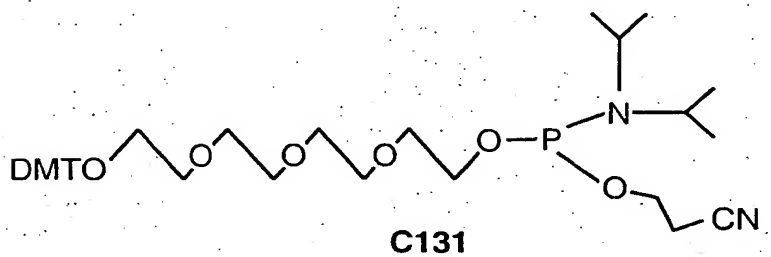


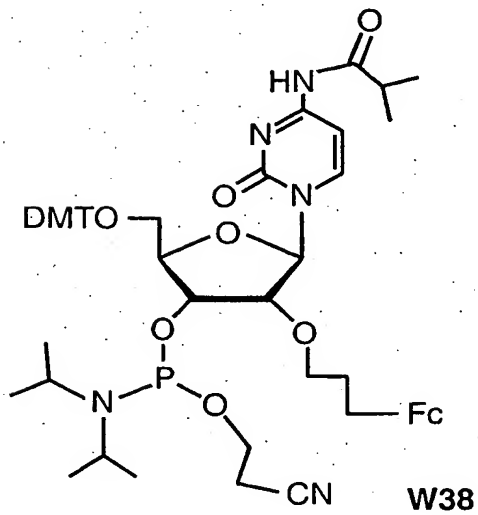
FIG._15K

N11



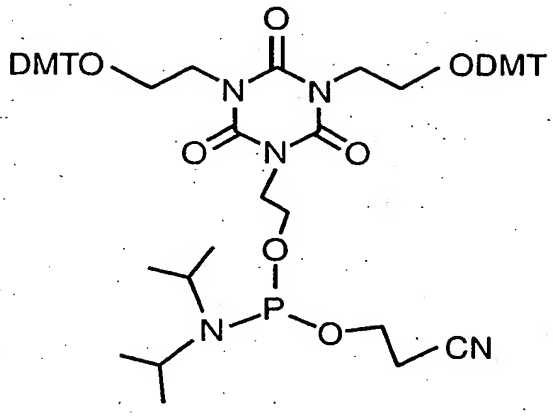
C131

FIG._15L



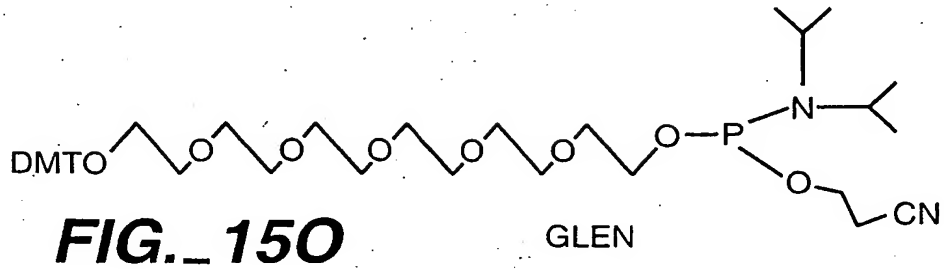
W38

FIG._15M



BRANCHING

FIG._15N



GLEN

FIG._15O

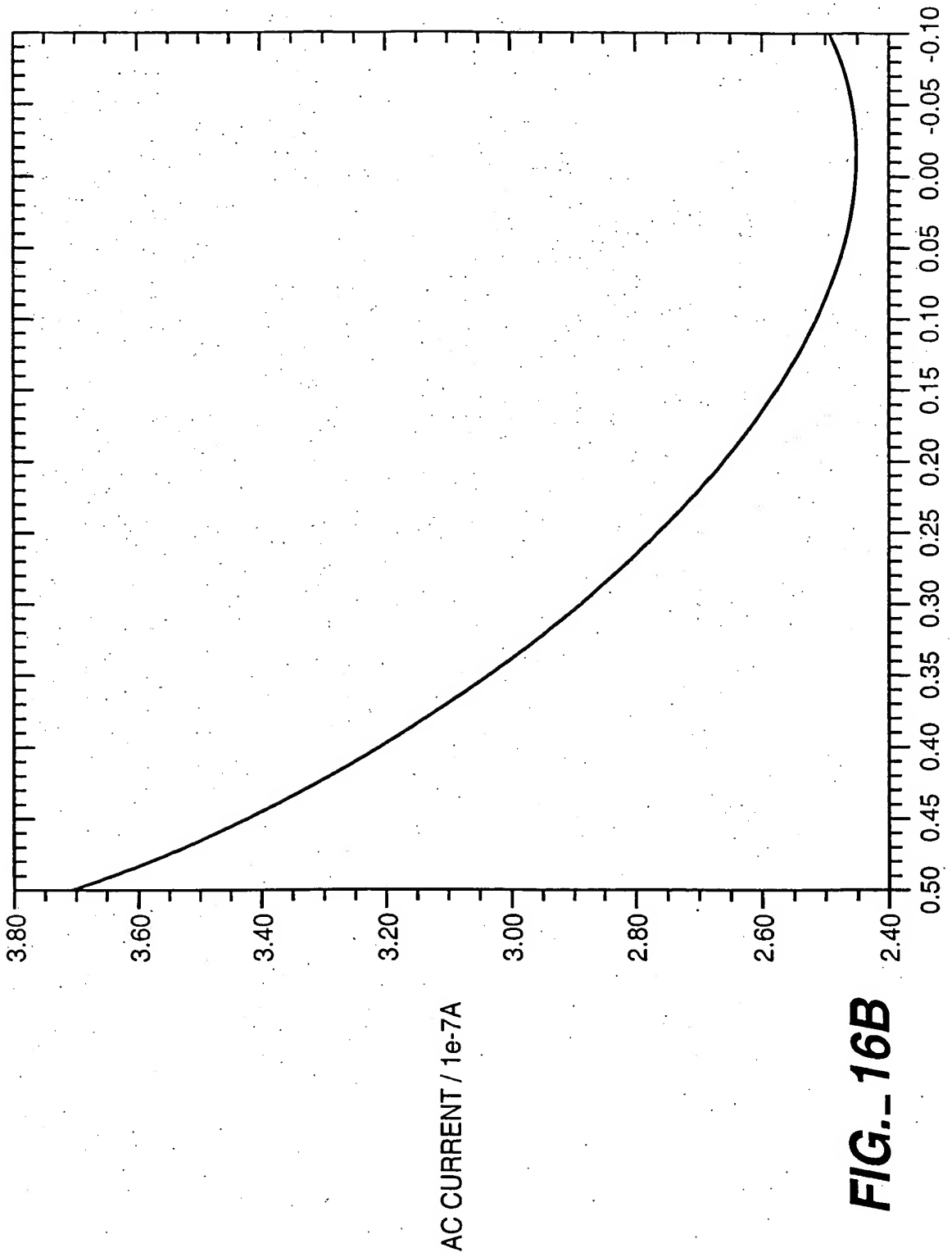
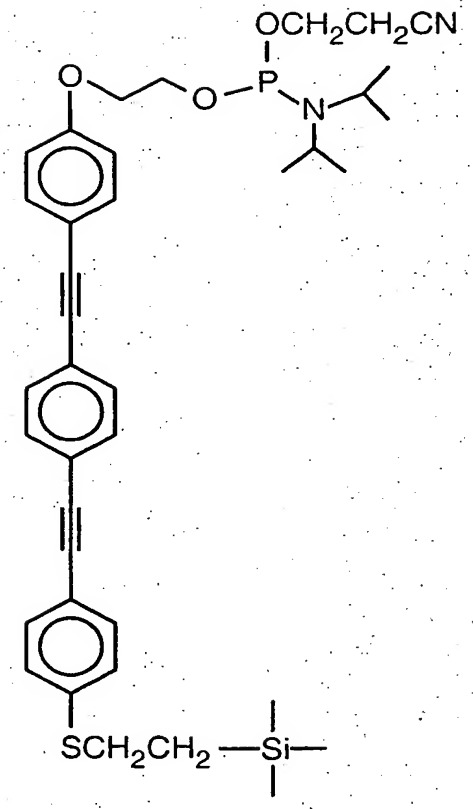
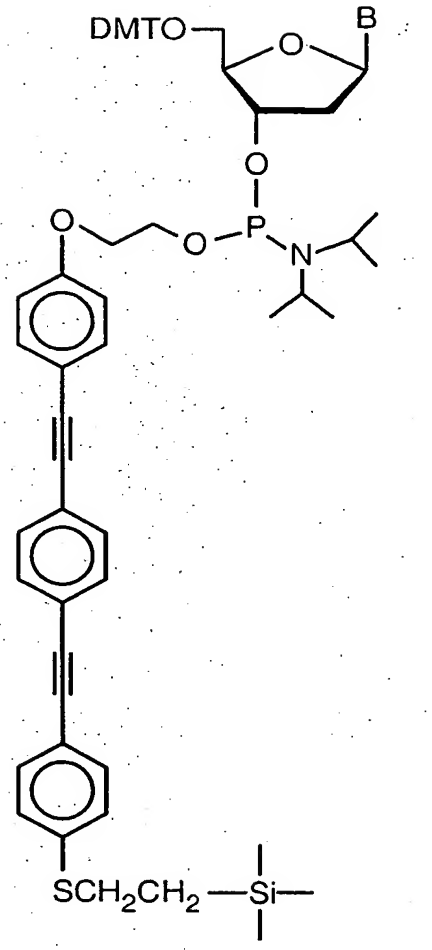


FIG._16B



5' - ATTACHMENT

FIG._18A



ANY POSITION ATTACHMENT

FIG._18B

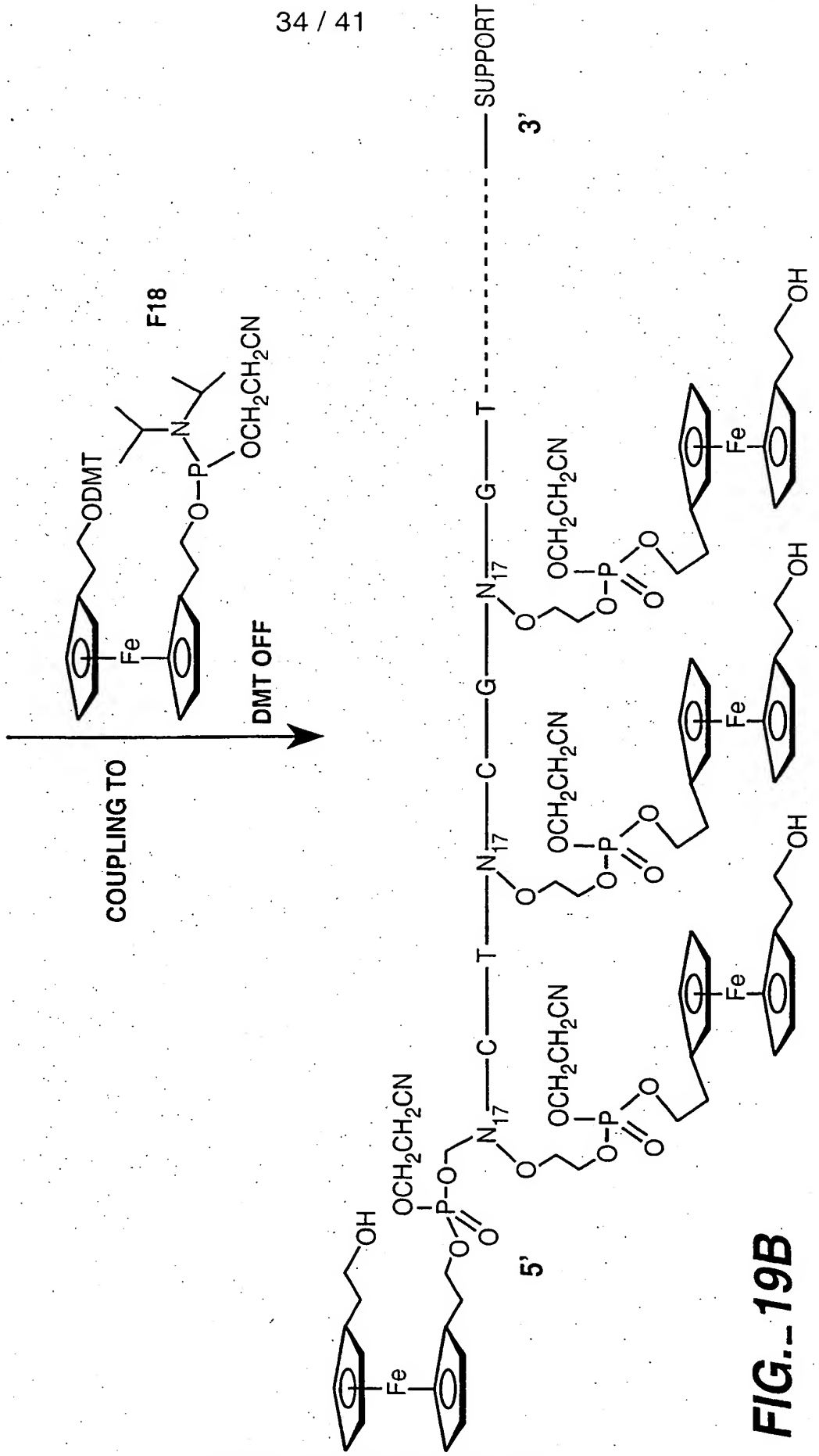


FIG._19B

FIG._20

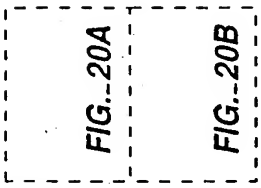
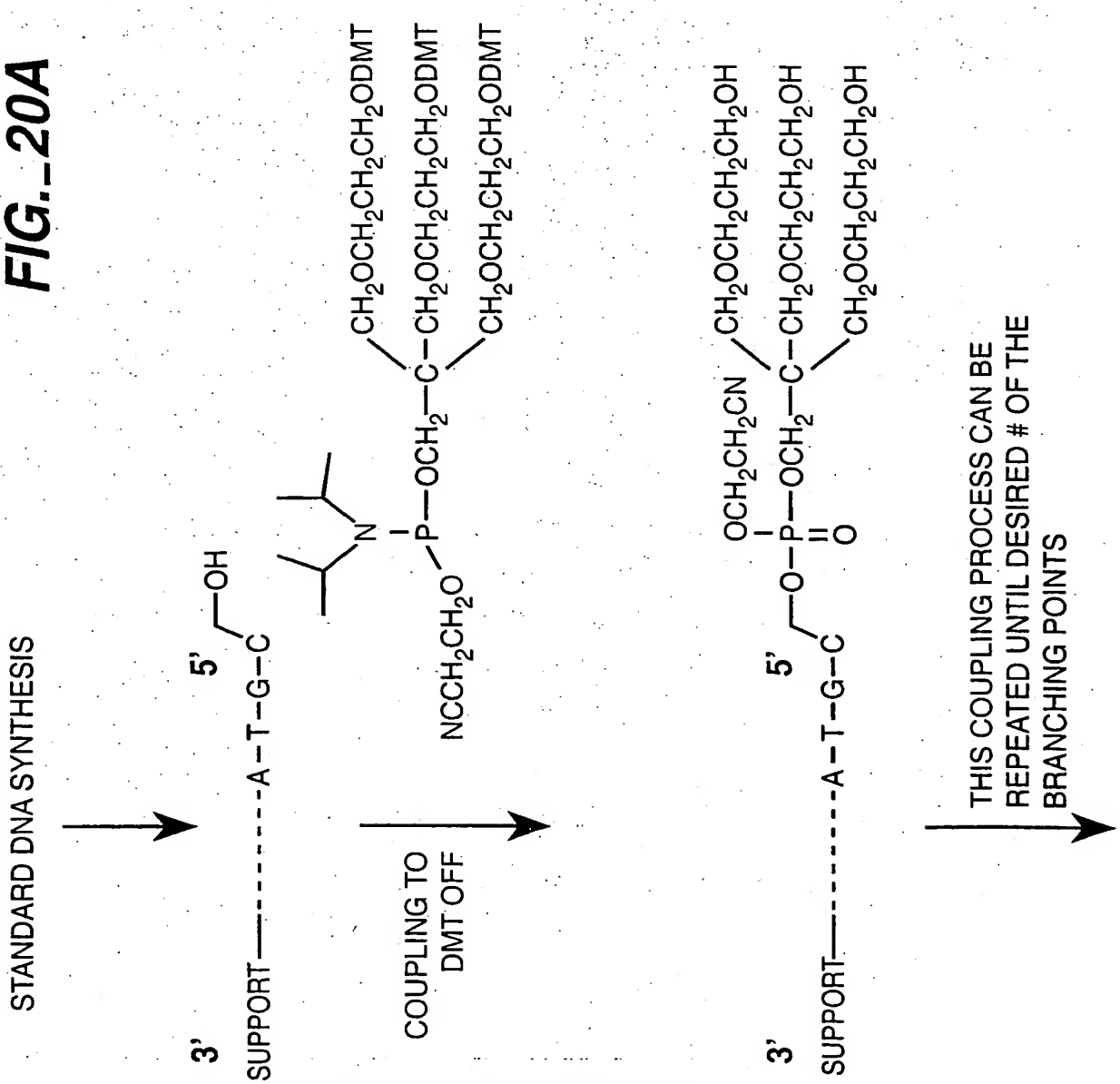


FIG._20A



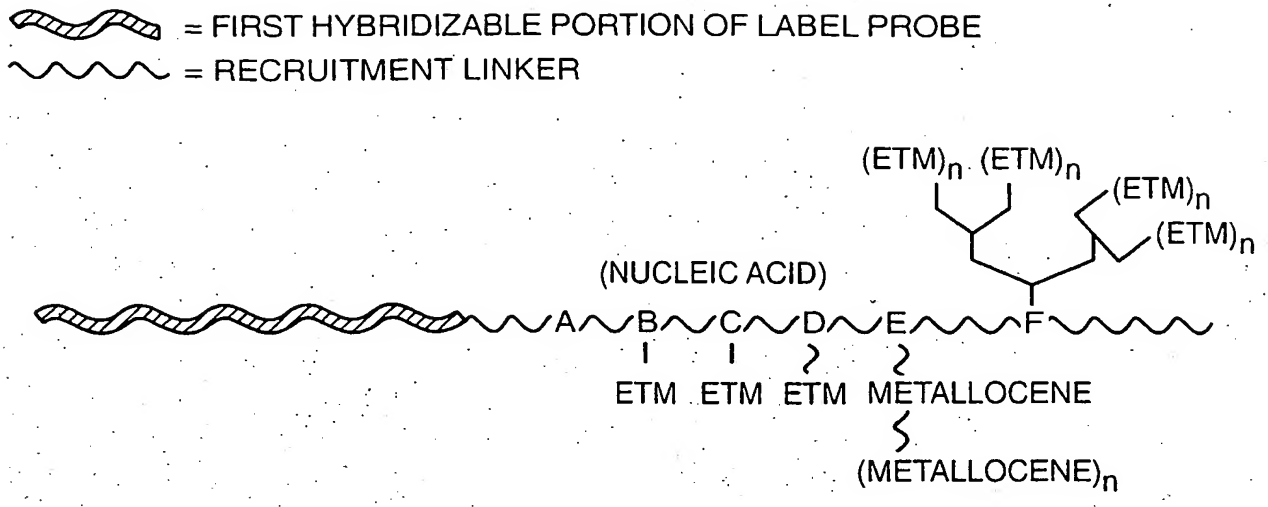


FIG._21A

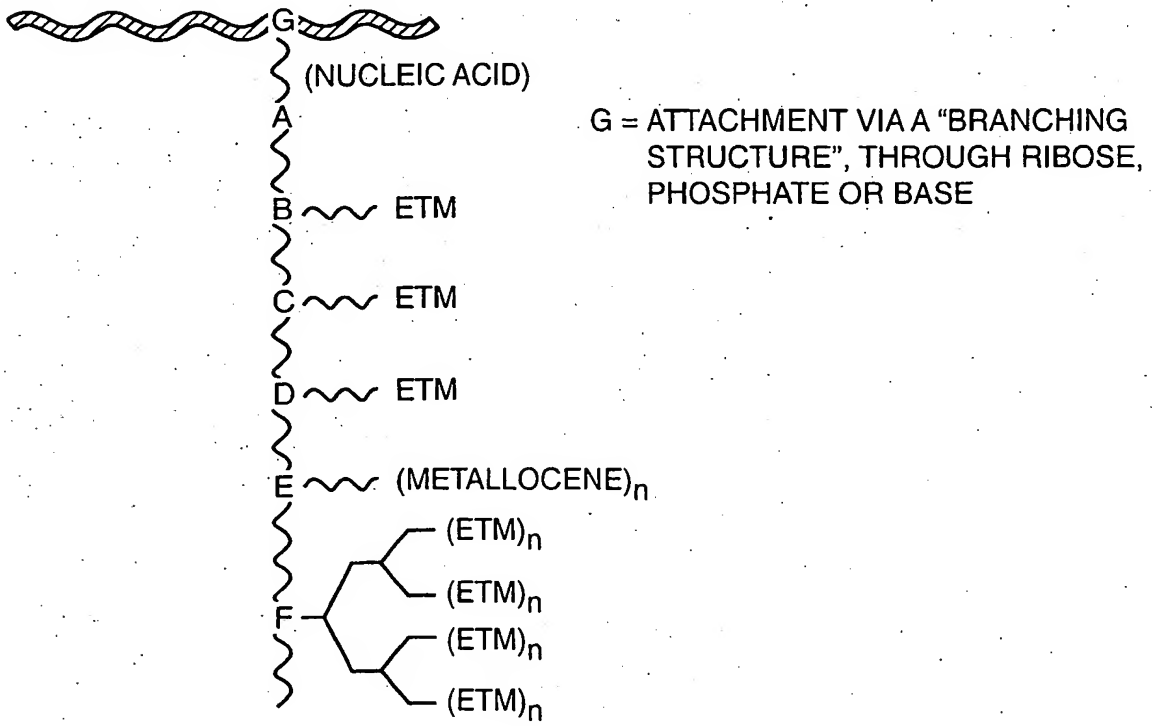


FIG._21B

FIG._22

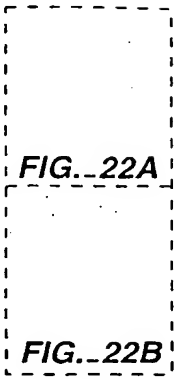


FIG._22A

D179
5' – A(C15)CCTGGTCTTGACATCCACGGAAGGCGTGGAAATACGTATTCGTGCCTA – 3'

D309 (Dendrimer)
5' – (W38)(Branching)(Branching)CATGGTTAACGTCAATTGCTGCGGTTATTAA – 3'

D295
5' - (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)CCCATGGTTAGACTGAATTGCTGCGGTTATTAA – 3'

D297
5' - (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)TATGCTCTTGATGGTGCTGTGGAAATCTACTGG – 3'

D298
5' – (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)ATGGTGCTGTGGAAATCTACTGG – 3'

D296
5' - (N6)G(N6)CT(N6)C(N6)G(N6)C(N6)TGACTGAATTGCTGCGGTTATTAA – 3'

D112
5' – CTTCCGTGGATGTCAAGACCAGGAU – 4 unit wire (C11) – 3'

D94
5' – ACCATGGACACAGAU – 4 unit wire (C11) – 3'

D109
5' – CTGCGGTTATTAACU – 4 unit wire (C11) – 3'

2Tar
5' – TAG GCA CGA ATA CGT ATT TCC ACG ATA AAT ATA ATT AAT AAC CGC AGC AAT TGA CGT ATA AAG CTA TCC CAG TAG ATT TCC ACA GC – 3'

D349
5' – A(C15)C (C15)GT GTC CAT GGT AGT AGC TTA TCG TGG AAA TAC GTA TTC GTG CCT A – 3'

D382
5' – (Y63)G(Y63) CT(Y63) C(Y63)G (Y63)C(Y63) CCC ATG GTT AGA CTG AAT TGC TGC GGT TAT TAA – 3'

D383
5' – (Y63)G(Y63) CT(Y63) C(Y63)G (Y63)C(Y63) CCC ATG GTT AGA CTG GCT GTG GAA ATC TAC TGG –3'

D468
5' – (N6)G(N6) CT(N6) C(N6)G (N6)C(N6) (glen)(glen)(glen) CTT TAC TCC CTT CCT CCC CGC TGA AAG TAC – 3'

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 99/10104

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N27/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	UTO Y ET AL: "Electrochemical analysis of DNA amplified by the polymerase chain reaction with a ferrocenylated oligonucleotide" ANALYTICAL BIOCHEMISTRY, vol. 250, no. 250, 1997, pages 122-124 124, XP002106964 ISSN: 0003-2697 ---	1-21
A	WO 86 05815 A (GENETICS INT INC) 9 October 1986 (1986-10-09) the whole document ---	1-21
A	WO 96 40712 A (CALIFORNIA INST OF TECHN) 19 December 1996 (1996-12-19) the whole document ---	1-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1999	19/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

ɔrmation on patent family members

Intern nal Application No
PCT/US 99/10104

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WO 9820162 A	14-05-1998	AU 5196798 A	29-05-1998
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